Causes of sex-different mortalities in birds: pathogens and immunocompetence

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Causes of sex-different mortalities in birds: pathogens and immunocompetence

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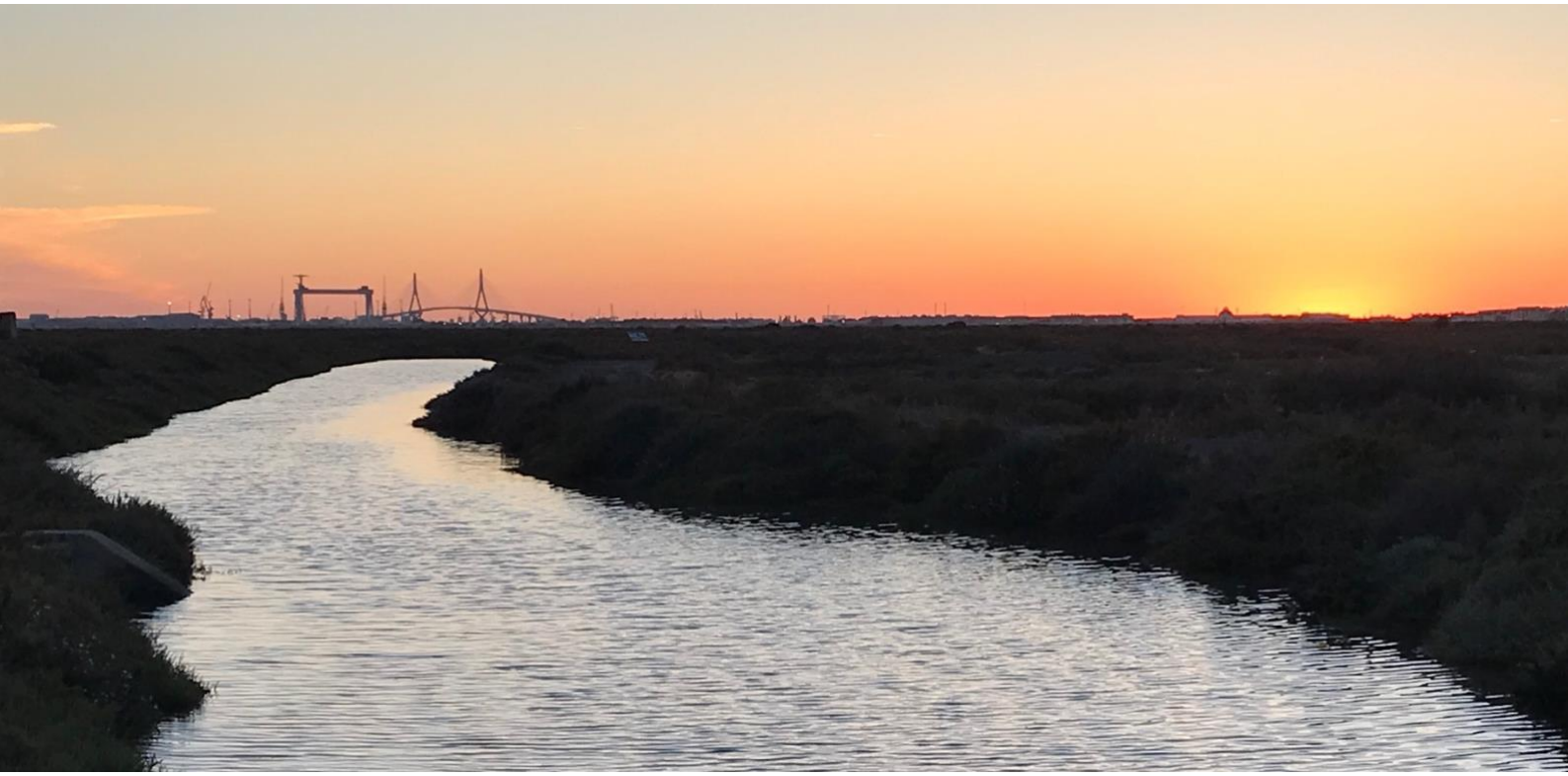
to my parents, María and José



Cádiz Bay, Spain 2019 – José O. Valdebenito

"*Buy the ticket, take the ride…*"

Hunter S. Thompson

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Summary

Males and females often exhibit different behaviour in reproduction (termed sex roles), which is believed to have driven sex differences in many aspects of the animals' biology, such as morphology and physiology. A central consequence of this divergence in behaviour are sex differences in mortality that, despite being widespread across animals, often their causes are unknown or ignored. Birds are charismatic vertebrates with remarkable features that make them ideal candidates for the study of breeding system evolution, such as diverse parental care, and flexible mating systems. Importantly, in most birds, females have higher mortality rates than males. Several life-history variables have been associated with female-biased mortality, but the proximal causes of this sex bias are widely unknown. The main objective of this dissertation is to explore how pathogens and the immune system are involved in sex-specific mortality in birds. My PhD used both single- and multi-species approaches to evaluate different aspects of macro- and micro-parasite infection and of the avian immune system. By using a small shorebird, the Kentish plover (*Charadrius alexandrinus*), as an ecological model organism, I show that bacterial infection on island and continental plover populations are not different, although females have higher infection rate than males. In a follow-up comparative study, I show that the prevalence of gastrointestinal and blood parasites does not differ between the sexes and parasite prevalence is not associated with sex-specific mortality across 138 species of birds. Then, taking a immunological approach, I used samples from wild populations of Black-winged stilts (*Himantopus Himantopus*) and Kentish plovers, to show that these two species lack sexual differences in agglutination and lysis titres, which partly follows predictions from demographic variables, although transcriptomic data of two different populations of Kentish plovers do show a male bias in expression of immune genes in the brain. Finally, in a comparative study across 41 wild bird species I showed that sex-specific immune response depends on the breeding status of the birds, where males tend to be more immunocompetent than females during the breeding season. Taken together, my PhD work suggests that the relationships between parasite prevalence, immune-responses and sex-different mortalities are not as simple as one would hypothesise. Finally, I discuss the contribution of my studies to the understanding of sex-specific mortality in birds, considering limitations and potential future studies.

Chapter 1 | Introduction

One of the fundamental patterns in animal social behaviour is that females tend to be the caring sex, whereas males compete for access to females (Queller, 1997; Henshaw et al., 2019). This is usually termed as 'conventional sex roles.' Anisogamy (different gamete sizes between the sexes) is thought to, at least partially, explain the fundamental origin of divergence of sex roles as gamete dimorphism defines the sexes; prior to mating one sex specialises on providing nourishment for their gametes whiles the other sex focuses on motility of their gametes (Parker et al., 1972; Lehtonen et al., 2016; Henshaw et al., 2019). The initial asymmetry in pre-mating parental investment (eggs *versus* sperm) is assumed to promote divergence in post-mating parental investment (parental care). Bateman (1948) was the first to recognise the prominence of anisogamy in sex role determination by proposing that anisogamy ultimately drove the male bias in sexual selection that is often observed in sexually reproducing animals. Based on field observations, Charles Darwin argued that males are typically eager to copulate, whereas females are choosy about whom to mate with (Darwin, 1871); together the Darwin-Bateman paradigm is the most commonly invoked concept to explain conventional sex roles (Dewsbury, 2005; Janicke et al., 2016).

However, there are abundant exceptions to the above pattern. These are labelled as sex-role reversal and consist of animals where males contribute more to care than females, and females compete for males. Sex-role reversal is widespread in nature, occurring in insects, fish, amphibia and birds (Clutton-Brock, 1991; Eens & Pinxten, 2000). These behavioural exceptions were first explained by recurring to ecological or life-history drivers, although empirical studies failed to support these hypotheses (Oring, 1986; Clutton-Brock, 1991; Bennett & Owens, 2002). More recent studies now link sex-role reversal to variations in adult sex ratios (ASR) and mating competition, mating systems and parental behaviour, because ASR directly affect the number of available males and females in the social environment (McNamara et al., 2000; Kokko & Jennions, 2008; Liker et al., 2013). In spite of these recent works, the association between ASR and sex role behaviour has remained controversial (Fromhage & Jennions, 2016).

Sex-specific parental contribution varies greatly in nature, with strong differences between and within animal groups. In mammals, male-only care is completely absent, and females care alone in about 90% of species (Royle et al., 2012; West & Capellini, 2016). In birds, 90% of species show biparental care, with cooperative breeding being part of this portion (about 9% of species, Cockburn, 2006; Remeš et al., 2015). But on average, female birds invest more heavily than males (e.g. Møller & Birkhead, 1993; Schwagmeyer et al., 1999). In reptiles with parental behaviour, care is provided either by the female or by both parents (Reynolds et al., 2002). In amphibians, male-only and female-only care has evolved equally, while biparental care occurs at a low level (Beck, 1998; Summers et al., 2006; Summers et al., 2007; Vági et al., 2019). In fish, the ratio of genera with male-only to biparental to female-only care is 9:3:1 (Reynolds et al., 2002), but which sex provides the bulk of care varies widely among families (e.g. Goodwin et al., 1998; Goodwin et al., 2002;

Mank et al., 2005). Finally, in invertebrates with parental care the majority have female-only care, biparental care is uncommon, and male-only care is rare (Zeh & Smith, 1985). In arthropods, for example, male care has only evolved in eight independent lineages (Tallamy, 2000). Thus, from the preceding descriptions of male and female reproductive roles, it is difficult to imagine a single ecological or life-history reason why some organisms exhibit conventional sex roles whereas others are sex-role reversed.

1.1 Sex-specific mortality

With the polarisation of sex roles between males and females, selection favoured traits that independently optimise breeding success of each sex. Thus, in the conventional picture, males may increase body size or develop ornamentation that would help in fights against other males while competing for females, whereas females may benefit from developing conspicuous coats to go unnoticed in presence of predators while nesting (Figuerola & Green, 2000). Although not all species present such radical sex differences in traits, most animals do present at least some differences in sex roles either at pre-mating or post-mating. These sex differences might in turn impose direct increase in sex-specific risks of mortality due to, for instance, aggression (Liu et al., 2017), or in indirect fashion such as higher risk of parasite infection (van Oers et al., 2010) (**Fig. 1.1**). In fact, sex differences in mortality are widespread in nature and have been reported across all major animal taxa (e.g. Rasgon, 2012; Eberhart-Phillips et al., 2018; Honeycutt et al., 2019; Lemaître et al., 2020). Whether males or females die more depends on the species in study and the causes of this are often unclear or ignored. Importantly, sex differences in mortality may alter the proportions of the sexes in the adult population and thus are considered as one important driver of ASR variation (Székely et al., 2014). Furthermore, sex-specific mortality also has implications for population dynamics, risk of extinctions and biodiversity conservation because the number of females impacts on population growth (Székely et al., 2014 and references therein; Eberhart-Phillips et al., 2017).

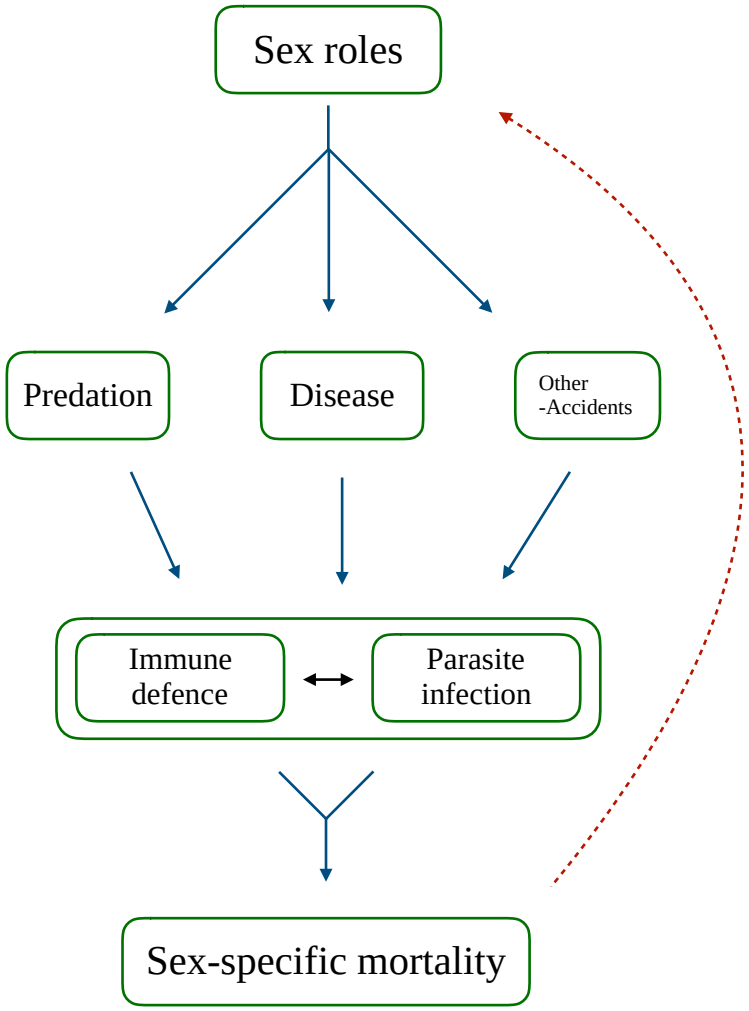

Figure 1.1 Logical framework that underpins the dissertation: the main causes of mortality in birds may be importantly determined by sex-different immune defences and parasite infection. The red dashed line denotates a feedback loop where sex-specific mortality will affect the pool of adult individuals in a population (ASR) that in turn might influence sex roles.

1.2 Sex difference in pathogens

A pathogen may be defined as any microorganism or agent able to cause disease, such as a virus, bacterium, parasite, fungus or others. These microorganisms are foreign to the host and are acquired during its lifetime. After conception, animals develop in a nearly sterile environment and start acquiring most of their microbiota during or immediately after birth (Dietz et al., 2019; Stinson et al., 2019), either directly from the environment, like in birds (Grond et al., 2017), or from the birth canal as in most mammals (de Goffau et al., 2019; Shao et al., 2019). Differences in lifestyles between males and females may create different rates of exposure to pathogens that could be in part responsible for the sex differences in pathogen burdens in natural populations (e.g. Poulin, 1996; McCurdy et al., 1998; Moore & Wilson, 2002; Poiani & Gwozdz, 2002; White et al., 2011). One recurring example of increased sex-specific exposure to blood parasites includes nesting birds, where the

sex that provides most of the pre-hatching parental care (i.e. sitting on eggs inside the nest) will experiment increased risk in attacks of mosquitoes (Culicidae), biting midges (Ceratopogonidae), or louse flies (hippoboscidae) (Korpimäki et al., 1993; van Oers et al., 2010), the main vectors of avian malaria parasites (Lehane, 2005). Another example of sex differences in pathogen burden relates to sexual size dimorphism and argues that the larger sex provides greater space and more resources for parasites, thus representing less ephemeral habitat patches than the small-bodied sex (based on the island biogeography theory, MacArthur & Wilson, 1967). Also, a larger animal has higher energetic demands, which may translate into increased contact with gastrointestinal parasites through food consumption (Kamiya et al., 2014).

Males and females also differ greatly in their physiology. In vertebrates, sex differences in physiology are triggered prematurely by exposure to different concentrations of sex hormones (i.e. oestrogens and androgens) secreted by their gonads during embryonic development, ultimately influencing many metabolic pathways thus defining the sexes as known (Kobayashi et al., 2018). The immunomodulating effect of sex hormones has been exploited to explain differences in parasite burden (e.g. Klein, 2004); however, recent evidence challenges this previously accepted association (Foo et al., 2017; see next section).

Social behaviour and mating strategy could also affect pathogen infection rate. The sex-specific social structure in highly social animals could dramatically affect pathogen transmission when compared to the ones that live in solitude (VanderWaal et al., 2014; Sah et al., 2018). In many mammals such as the Northern fur seal (*Callorhinus ursinus*), their breeding social structure is divisive and allocates individuals into two broad groups: (i) harems with one male and many females, and (ii) groups of young males that were excluded from the previous to join bachelor herds (Gentry, 1998). In Northern fur seals, pathogen transmission rate will be importantly constrained by the social group infected and whether the pathogen is sexually transmitted. The mating system, a life-history trait that predicts the behavioural patterns of sexual interactions in animals, can also influence pathogen acquisition because promiscuity entails higher risk of infection than monogamy.

However, the sex-specific prediction of infection varies according to the specific animal system in study. In birds, theoretical models studying the prevalence of sexually transmitted infections (STIs) and their association with mating systems predict that, at the population level, the sex with the higher variance in mating success should have lower prevalence of infection (Thrall et al., 2000; Ashby & Gupta, 2013). However, although these theoretical models assume a polygynous population and balanced adult sex ratios –not a general occurrence in the wild (Halimubieke et al., in review; Donald, 2007; Székely et al., 2014; Eberhart-Phillips et al., 2018)– the predictions of Thrall et al. (2000) and Ashby & Gupta (2013) in skewed adult sex ratios should hold, because at the population level, the sex with higher variance in mating success will proportionally have fewer sexual interactions and thus lower prevalence of STIs. Furthermore, the few available empiric studies that have addressed this topic in the wild confirm a cost of bacteria transfer associated to multiple mating compared to monogamy (Poiani & Gwozdz, 2002; White et al., 2011).

1.3 Sex difference in immunity

The purpose of the immune system is to distinguish between the hosts' healthy tissue and exogenous elements such as parasitic worms. Because the host is in constant interaction with the environment, the immune system is continuously challenged by exogenous agents, many of them of pathogenic potential that, if not controlled, could result in lethal consequences for the host (Abbas et al., 2015). The immune system is broadly divided into innate immunity and adaptive immunity. The innate immunity, primitive in origin, corresponds to an immediate and unspecific response and is broadly present across animals, whereas the adaptive immunity is slower to develop, more specific, and restricted only to vertebrates (Buchmann, 2014; Abbas et al., 2015).

Different immunocompetence between males and females is common in vertebrates, historically associating those sex differences to the immune suppressive effect of androgens in males and the immune-enhancer influence of oestrogens in females (Klein & Roberts, 2010; Furman et al., 2014; Klein & Flanagan, 2016). However, these studies have primordially been centred on laboratory animals and humans. While more broad views have challenged the immune-modulatory effect of sex hormones in wild populations as two independent meta-analyses found inconsistent results (Roberts et al., 2004; Foo et al., 2017). Nevertheless, research in mammals has repeatedly supported an important influence of sex hormones on immunity (see Zuk, 2009; Klein & Flanagan, 2016; Smyth et al., 2018).

Because the immune system is complex and requires immense allocation of resources to function, hypotheses linking energetic investment to sexual selection and mating systems have been proposed to explain differences in immune function, particularly in birds. The economy of energetic investment should emphasise allocation of resources to traits that maximise individual fitness of the sexes. For example, if investing in longevity will improve females' reproductive success, more resources should be allocated into immunity (Rolff, 2002). Generally under polygamy (polygyny), males are the competing sex, manifested as behavioural traits (e.g. aggression; territoriality), larger body size (relative to females), or ornamentation, while in monogamous species, these differences between the sexes tend to be minimal, and thought to be due to lower forces of sexual selection acting on the sexes (Zuk & McKean, 1996; Rolff, 2002; Fairbairn et al., 2007; Janicke et al., 2016).

Zuk (1990, 1994) proposed that sex differences in immune capacity may be driven by the different ways the sexes achieve reproductive success. Although this idea has been modestly addressed, shows a rather adequate consistency towards no sex bias in immune capacity in monogamous populations, and immune sex biases in polygamous ones. For example, Blue tits (*Cyanistes caeruleus*) and Tree sparrows (*Passer montanus*), two monogamous passerines species, showed no sex differences in humoral immune function (Råberg et al., 2003; Lee et al., 2006). While sex differences are found in polygamous species as in Red-winged blackbirds (*Agelaius phoeniceus*) and the Red junglefowl (*Gallus gallus*) where males showed lower immune function level than females (Zuk, 1996; Hasselquist et al., 1999). Unfortunately, the studies available are few and tend to focus on monogamous species. A recent meta-analysis in birds showed no sex bias in immunity, which follows up several mixed and inconclusive results in this topic (Kelly et al., 2018). Nevertheless, Kelly et al. (2018) had considerable limitations, for instance they pooled estimates of innate and

adaptive immunity together, they also pooled different age classes and different types of immune assays, so that the potential impact of important variables like timing in the season and mating system may have masked sex-different immune responses.

Research in mammals has shown that important sex differences in immunocompetence consistently find females as the stronger sex (Zuk, 2009; Klein & Flanagan, 2016; Natri et al., 2019), although most of these studies have been conducted in humans and laboratory animals. For instance, women with acute HIV infection have 40% less viral RNA in their blood than men (Griesbeck & Altfeld, 2015). In most countries, tuberculosis notification is twice as high for men than women, suggesting that for tuberculosis, being male is a risk factor (Horton et al., 2016). Furthermore, the recent COVID-19 pandemic showed significantly higher mortality rate in men infected with the virus than in women in spite of approximately equal infection rates of males and females (GlobalHealth5050, 2020). There are various hypothesised explanations for these sex differences, although in humans, the explanations often invoke proximate mechanisms such as changes in sex steroid concentrations (discussed above) and X chromosome diploidy, based on the fact that the X chromosome contains relatively large immune coding genes compared to the Y chromosome (Case et al., 2012; Case et al., 2013). Nevertheless, in most wild animals, the causes of sexual differences in immune function are still largely unknown.

1.4 Why study birds

Birds have been source of wonder and admiration in many cultures throughout history of mankind. Their ability to fly is arguably the main feature that firstly caught our attention as kids: how they can do that? –one, naively, might have asked. Perhaps this partly explains the reason why birds are one of the best studied vertebrates, along with the fact that the majority of birds are diurnal; a clear advantage compared to other vertebrates such as mammas that are mostly nocturnal. Birds are important players in ecosystems because survival of many plant species depends on pollinators such as hummingbirds, or the spread of seeds by other fruit-eating birds. Birds of prey can also be crucial from an epidemiological perspective because they are important pest controllers by feeding on animals of zoonotic potential such as rodents.

Importantly, birds show great range of sexual dimorphism, where males and females may be indistinguishable from one another in species like the Jackdaw (*Corvus monedula*), to cases where males can be up to three times heavier than females like in the Great bustard (*Otis tarda*). Some birds also present reverse sexual dimorphism (females larger and/or brighter than males). Furthermore, their parental care duties and mating system behaviour varies dramatically between species. This rich diversity of traits between the sexes places birds as a unique group to study the origin of their sex differences in mortality, as well as its relationship to sex differences in immune variables and pathogen burden. In fact, birds are the group of vertebrates with the largest and most detailed data available on mating system and sex roles, a clear advantage when aiming to untangle universal patterns.

1.5 Dissertation objectives

The main objective of my dissertation is to investigate the extent of sex differences in pathogen burden and in immunity in birds, and test whether these sex differences may be linked to sex differences in mortality. To understand the complex relationship between sex roles, parasites and immunocompetence, my dissertation has two main objectives; these objectives are addressed using single- and multi-species (comparative) approaches:

(i) Investigate whether micro- and macro-parasites of known biological relevance are present at different rates of infection in male and female wild birds.
(ii) Determine whether males and females differ in various aspects of their immune system in wild birds.

The chapters presented here address the above question by, first, investigating the sex-specific prevalences in micro- and macro parasites in birds, to then shift the approach towards the host, by investigating parameters of the immune system using both single- and multi-species methodologies. My main study organisms are birds, where I carried out several studies using shorebirds as model organisms. Shorebirds (Charadriidae: plovers, sandpipers and allies) are highly suitable for evolutionary studies since they present broad behavioural and ecological diversity in mating and parental strategies, thus being an excellent model systems to investigate sexual selection, breeding systems and sex roles (Székely & Reynolds, 1995; Thomas et al., 2007; Székely, 2019).

The first two chapters focus on parasites. In **Chapter 2** I centre on a well-studied shorebird, the Kentish plover (*Charadrius alexandrinus*), and investigate the prevalence of three important bacteria on Kentish plover populations from the continent and islands, considering sex-specific infection and its impact on body condition. Although many processes could result in different parasite burden in the sexes in birds, it was not clear whether parasite prevalence could substantially contribute to sex-specific mortality. Such an assessment was long overdue, given that the last reviews on this topic were published more than 20 years ago (Poulin, 1996; McCurdy et al., 1998). Therefore, in **Chapter 3** I carry out a meta-analysis and comparative analysis methods to investigate the effect of sex-specific gastrointestinal and blood parasites on mortality across 138 species of birds.

In the following three chapters I focus on immune capacities since the immune system is critical for pathogen defence and hence host survival. The immune system is energetically expensive and has been proposed to be traded-off against a number of life-history variables in order to maximise reproductive success. Thus, in **Chapter 4** I tested the methodology to assess the sex-specific strength of innate immune function in two wild shorebird populations (Kentish plovers and Black-winged stilts, *Himantopus himantopus*). Whereas in **Chapter 5**, I compared the immune gene expressions in the brains of male and female Kentish plovers to assess sexual dimorphism in gene expression. Whilst substantial research has been conducted on immune

defences of males and females in wild birds, the results are inconsistent across species. To seek general patterns, in **Chapter 6** I carry out a multi-species analysis of the sex-specific effect of breeding status on eight immune parameters using data from 41 bird species.

In **Chapter 7** I summarise the results in the context of sexual differences in mortality in birds, discussing possible limitations of the various research approaches. Finally, I propose unexplored areas that have potential in further explaining such sex differences in nature.

I would like to note that I acknowledge the variety of interpretations that sex roles could entail (Schärer et al., 2012), and it is not my objective to discuss these differences here. In this dissertation I have adopted the approaches presented by Janicke et al. (2016) and Jennions & Fromhage (2017).

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Chapter 2 | Association of insularity and body condition to cloacal bacteria prevalence in a small shorebird

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RESEARCH ARTICLE

Association of insularity and body condition to cloacal bacteria prevalence in a small shorebird

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Abstract

Do islands harbour less diverse disease communities than mainland? The island biogeography theory predicts more diverse communities on mainland than on islands due to more niches, more diverse habitats and availability of greater range of hosts. We compared bacteria prevalences of *Campylobacter*, *Chlamydia* and *Salmonella* in cloacal samples of a small shorebird, the Kentish plover (*Charadrius alexandrinus*) between two island populations of Macaronesia and two mainland locations in the Iberian Peninsula. Bacteria were found in all populations but, contrary to the expectations, prevalences did not differ between islands and mainland. Females had higher prevalences than males for *Salmonella* and when three bacteria genera were pooled together. Bacteria infection was unrelated to bird's body condition but females from mainland were heavier than males and birds from mainland were heavier than those from islands. Abiotic variables consistent throughout breeding sites, like high salinity that is known to inhibit bacteria growth, could explain the lack of differences in the bacteria prevalence between areas. We argue about the possible drivers and implications of sex differences in bacteria prevalence in Kentish plovers.

Introduction

Understanding how biological diversity stablishes and evolve has been a tradition in modern ecology [1, 2]. Insights of historic and contemporary research have led ecologists to develop a number of biodiversity theories that are intended to help us predict biodiversity in a given space and/or time. According to the theory of island biogeography, landscape structure shapes species' abundance, where species richness increases as a function of the area sampled [3]. Along a gradient of ecosystems of increasing size, the number of species inhabiting those

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ecosystems will increase rapidly at first, but then the pace slows down for the larger ecosystems [3, but see limitations, 4]. Island biogeography theory has been mainly built upon the study of macroorganisms, with very little consideration towards the biogeography of microorganisms. In fact, whether microbial biogeography should be considered as a discipline has been subject of debate, because it has long been suggested that organisms smaller than 1 mm have a cosmopolitan distribution [5]. However, different studies have documented spatial and temporal structuration of microbial diversity [6, 7]. For example, positive taxa-area relationships have been found in free-living bacteria [8], as well as a reduction in bacterial diversity across islands of decreasing sizes [9].

Symbiotic organisms are in a close and necessary association with other organisms, through either mutualistic, commensal or parasitic associations [10]. This co-dependant interaction adds an important layer of complexity to the island biogeography theory, mainly for including variables from the host that could also be affected by insularity. For example, animals from islands have been proposed to have weaker immune defence, attributed to the founder effect during colonization and to island environments being relatively parasite poor (compared to the mainland) [3, 11, 12]. Examples of the latter include reduced prevalence and diversity of blood parasites and fewer feather lice species in Macaronesian blackcaps (*Sylvia atricapilla*) [13, 14] and reduced viral pathogen diversity and abundance in insular black-spotted pond frogs (*Pelophylax nigromaculatus*) compared to the mainland [15]. However, contrasting results could be found for other host species and microorganisms studied [16].

Studies investigating variation in microorganism prevalence, not microorganism diversity, in relation to area sampled have been considerably less common. Microorganism prevalence, here defined as the percentage of individuals of a population infected with a given microorganism, will depend importantly upon two variables: (i) the ability of the host to defend against the infection, and (ii) on the ability of the microorganism to infect the host. As mentioned before, insularity is thought to, at certain extent, shape immune function because after many generations exposed to low pathogen pressure and diversity, selection favours a reduction in the energy invested in maintaining a robust immune function [17]. However, changes in immune parameters in response to insularity are not as straightforward as initially thought, as Matson et al. [18] found that in bluebirds (*Sialis sialis*) the immune response was stronger in island than in the mainland. Lobato et al. [19] investigated two immune components in bird assemblages from two islands and mainland in Africa, finding that acquired immunity was lower on islands but no differences were seen in the innate immunity. The high microorganism diversity expected in mainland [9] increases the chances of hosts of encountering strains of microorganism of high virulence, that could rapidly spread out across a population and elevate prevalence at a given sampling time [20]. Also, the force of infection (the rate at which susceptible individuals become infected in a population) is expected to increase with population size in the case of having many susceptible hosts present in a large population at any given time, and because in this ecosystem the number of contacts between infected and susceptible individuals is likely to also increment [21–24]. Conversely, in simplified ecosystem (i.e. islands) the reduced pathogen pressure compared to mainland would suggest, in general, lower levels of pathogen prevalence than in the mainland [25–27]. However, to our knowledge just a handful studies have tested aspects of these predictions, and to date there is very little known on how prevalence of bacteria could be affected by insularity.

Here, we tested for the first time the influence of insularity on the transmission patterns of the bacteria *Campylobacter*, *Salmonella* and *Chlamydia* in four populations of Kentish plover (*Charadrius alexandrinus*). These bacteria are known for its importance in wildlife and public health, being usually commensal in poultry but a common cause of gastrointestinal and respiratory disease in wild birds [28, 29]. Kentish plover is a small shorebird, ideally suited for this

purpose because breeds all across Eurasia and Macaronesia (Fig 1), islands where they are year-round residents and represent populations genetically distinctive from the mainland [30]. We compared the cloacal bacteria prevalence of two island populations from Cape Verde and Canary Islands, and two populations breeding in continental Spain (Table 1). Because these

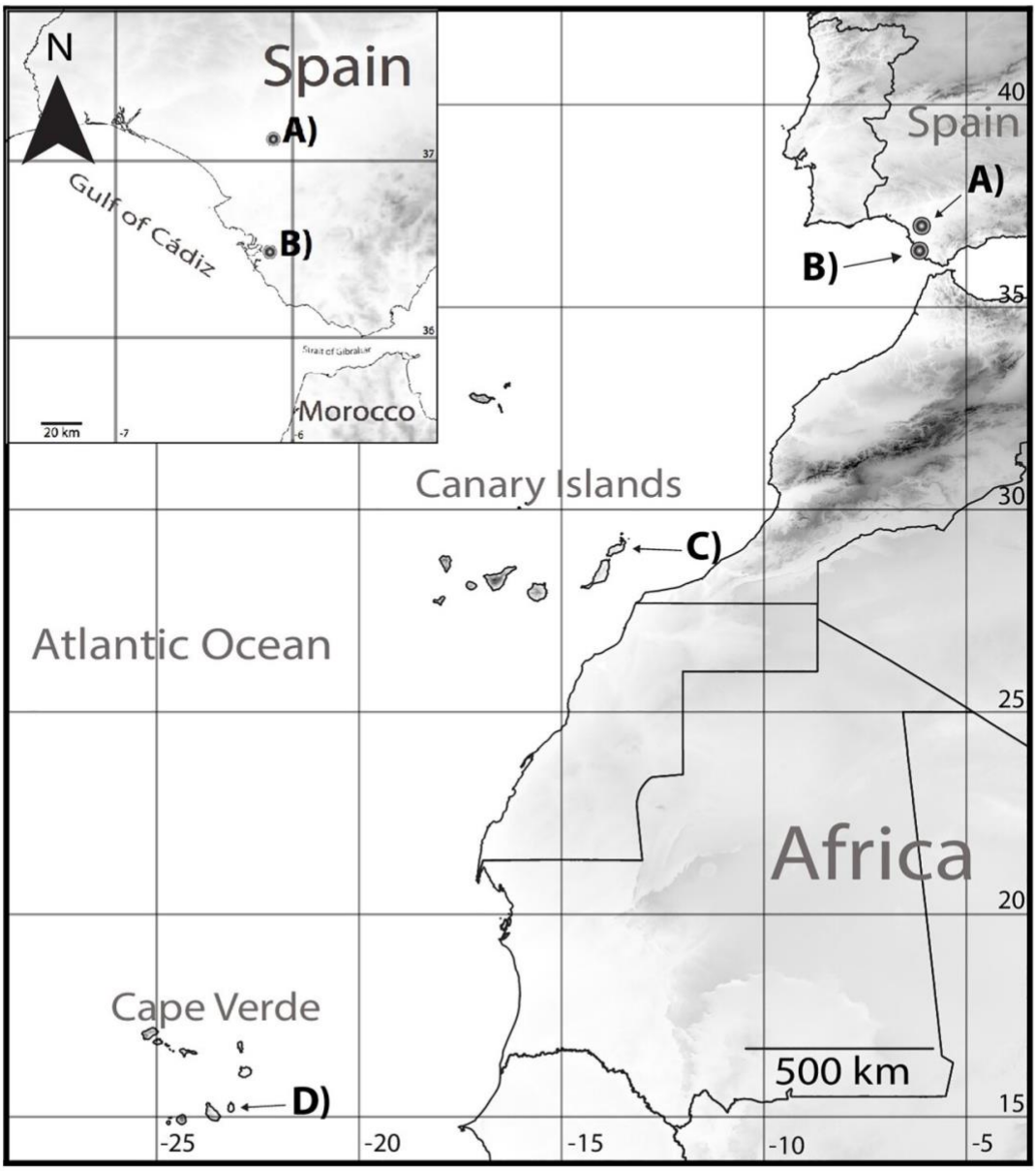

Fig 1. Sampling locations of the four Kentish plover populations. Two on mainland and two on islands: (A) Rice fields of Doñana, and (B) Salina la Esperanza, Cádiz, both in Spain; (C) Lanzarote, Canary Islands, and (D) Maio, Cape Verde.

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Table 1. Number of birds examined and infected in mainland and island populations of Kentish plover.

	Population	*n*	*Campylobacter*	*Chlamydia*	*Salmonella*	Pooled infection
Island	Maio, Cape Verde	88	2	5	10	17
	Lanzarote, Canary Islands	27	0	0	3	3
	Total	115	2	5	13	20
Mainland	Doñana, Spain	52	1	1	10	12
	Salina la Esperanza, Cádiz, Spain	52	1	4	10	15
	Total	104	2	5	20	27

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bacteria could provoke disease, we also investigated the effect of insularity and bacteria infection on body condition. Based on previous evidence, we predicted (i) a higher prevalence of infection in mainland than in insular populations; (ii) bacteria infection will negatively affect the host's body condition [31, 32]; and as possible consequence of the previous two, (iii) birds from islands will have better body condition than those from mainland [33]. Last, because cloacal transmission of bacteria seems to be asymmetrical between the sexes [see 34], we predicted (iv) females potentially having higher prevalences than males.

Materials and methods

Ethics

All necessary permits were obtained for the described field studies. Salina la Esperanza: permit granted by the University of Cádiz, Cádiz Bay Natural Park and Animal Health authorities in compliance with Spanish laws (number 2019-/2979/4202/Bc/EA 3619). Doñana: permit granted by the CSIC Ethics committee and Animal Health authorities in compliance with Spanish laws (number 2011_02 21/02/2012/77). Lazarote: permit granted by the Council of Land Usage, Sustainability and Security, Vice-Ministry of Environment, Canary Islands (number 2016/3646). Maio: permit granted by the General Directory of Environment, Cape Verde (number 33/2014).

Bird species and sampling locations

We captured, ringed, weighed and morphometrically measured breeding Kentish plovers from two different locations in mainland (southern Spain) and from two Macaronesian islands (Table 1, Fig 1). One mainland Kentish plover population was studied in the largest area of rice fields in Spain (36,000 ha) located in a reclaimed marshland behind Doñana National Park, Spain (37˚07'08.3"N 6˚06'33.7"W). Fieldwork was done in July 2015 at four sites during the peak of the breeding season. The other mainland population bred in a 35-ha saltpan in the Cádiz Bay Natural Park, Puerto Real, Spain (36˚30'36.0"N 6˚09'20.8"W). Fieldwork was conducted in April–June 2015. Among the island populations investigated, we studied Kentish plover on Lanzarote, Canary Islands (29˚03'36.1"N 13˚36'24.9"W). Lanzarote is the easternmost island of the archipelago, separated by approx. 120 km from North Africa and 1,000 km from the Iberian Peninsula. Fieldwork was conducted during the breeding season in April–June 2016, monitoring five sites around the island with different environments: saltpans, sandy beaches and semi-desert rocky areas. Lastly, we studied the Kentish plover population in Maio, Cape Verde (15˚09'16.6"N 23˚11'39.4"W), one of four Sotavento Islands in the archipelago, located at approx. 650 km from West Africa and 2,900 km from the Iberian Peninsula. Approximately 100–200 pairs bred in Maio around areas of saline lakes and saltpans of approx. 100 ha and surrounded by sandy shores. Three sites were monitored during September–November 2015. Kentish plovers present high breeding-site fidelity [35, 36] whereas during

winter the birds from mainland Europe move to SW Europe and W African [37]. Kentish plover from Maio and Lanzarote are year-round residents but eventually could move between islands, particularly in the population of Lanzarote (TS, and GT pers. obs). All field procedures complied with the laws and approved by the ethics committees of the corresponding countries.

Bacteria sampling and laboratory diagnosis

Our analyses were focused on three bacteria genera of renown importance for wildlife and public health. *Campylobacter* and *Salmonella* are gram-negative bacteria from the Enterobacteriaceae family and often found as commensal microbiota in avian hosts [38, 39]. Commensal strains result from bacterial adaptation to specific hosts [28, 40]. In poultry, many specific strains are recognised as commensal such as *Campylobacter jejuni* ST-104 (ST-21 CC) in broiler [29], but in Kentish plover it is unknown whether there are species-specific strains and to what degree these strains are commensals or can harm host health. Nevertheless, pathogenic strains like *Campylobacter lari* or *Salmonella typhimurium* have been associated with gastrointestinal disease in poultry and in wild birds [29, 30, 41] and are also a latent epidemiological problem causing foodborne disease worldwide [42]. Although these bacteria are typically acquired through an oral-fecal route by ingesting contaminated food or water, evidence shows transmission after copulation, either through direct cloacal contact or due to ingestion of bacteria during post-copulatory preening [43, 34]. *Chlamydia*, on the other hand, are sexually transmitted bacteria and species like *Chlamydia psittaci* may cause chlamydiosis in birds and the zoonosis psittacosis (if transmitted to humans by contaminated aerosols), both being a systemic disease often linked to mortalities [44, 45].

Bacteria sampling took place at capture by gently introducing a sterile cotton swab into the bird's cloaca. Swabs were stored in phosphate-buffered saline (PBS) buffer at -20ºC in the field, and then in the laboratory at -80ºC until further analysis [46]. DNA extraction was done using the Maxwell® 16 Buccal Swab LEV DNA Purification Kit following the manufacturer's protocol. Detection of *Campylobacter* was based on amplification of a DNA segment within the *flaA* short variable region (SVR) of *Campylobacter jejuni* or *C. coli*, according to Ridley et al. [47]. *Chlamydia* detection centred on amplifying the IGS region and domain I of 23S rRNA gene, following Nordentoft et al. [48]. *Salmonella* detection used primers specific for the *invA* gene, as described by Rahn et al. [49]. In brief, real-time PCR assays were conducted with 5 μL of 2 × Rotor-Gene SYBR Green PCR Master Mix, 7 μL of RNase-Free water, 1 μL of primers, and 2 μL of DNA extract. Thermal conditions for PCRs were as follows: initial activation for 10 min at 95ºC, PCR cycling for 15 sec at 95ºC, for 30 sec at 59ºC and for 30 sec at 72ºC (for *Chlamydia*) or 30 sec at 95ºC, for 15 sec at 54ºC and 20 sec at 72ºC (for *Salmonella*) for 45 times, and melting curve were obtained by lowering the temperature from 90ºC to 75ºC, descending by 0.3ºC each step. We used DNA from *Chlamydia psittaci* and *Salmonella typhimurium* as positive controls in each reaction plate. For *Campylobacter* cycling was for 10 sec at 95ºC, for 6 sec at 50ºC and for 6 sec at 72ºC for 35 times, and melting curve were obtained by lowering the temperature from 90ºC to 50ºC, descending by 2.2ºC each step. The positive controls used were *Campylobacter jejuni* and *C. coli*. Negative controls were included in each plate.

Statistical analyses

Our predictions were tested by running Markov chain Monte Carlo simulations for generalized linear mixed models using the R package 'MCMCglmm' [50]. Differences in insularity were tested by running four models: one for each bacteria type and one for the prevalence of the three bacteria combined (pooled bacteria infection). The models had bacteria infection

(binomial variable: infected/un-infected) as response variable, and insularity (binomial variable: island/mainland) and sex (binomial variable: female/male) as fixed factors as well as the two-way interaction between these two variables. Date of sampling (Julian date) and sampling site were added as random terms. Sampling site corresponded to the sites sampled within each location: four in Doñana, one in Salina La Esperanza, five in Lanzarote, and three in Maio. We used parameter expanded priors for the random effects (list(V = diag(1)*0.02, nu = 7)), and fixed effect priors for binary responses i.e. fixing the residual variance at 1 (list(V = diag(1), nu = 0.002, n = 1, fix = 1)). Models were run across 1,000,000 iterations with thin of 600 and a burn-in of 1,500. These values were determined based on model convergence and autocorrelation levels assessed through the Gelman-Rubin test [51], and trace graphs and the 'autocorr' function, both implemented in the R package 'coda' [52]. In all four models, the potential scale reduction factor was 1.01 or lower, which is below the threshold of 1.1 indicating good model convergence. Autocorrelation was also low, always below the threshold of 0.1 [50].

Body condition was estimated using the scaled mass index proposed by Peig and Green [53], consisting on standardizing body mass for a given size using a body linear measurement (here, wing length). This analysis was conducted only for the pooled bacteria prevalence due to the very low prevalences of *Campylobacter* and *Chlamydia* (see results). This model was run with a Gaussian error distribution and had body condition as response variable and infection status, insularity and sex as fixed factors and their two-way interactions. Date of sampling and sampling site were added as random terms. We used parameter expanded priors for the random effects (same as above) but inverse gamma priors (list(V = 1, nu = 0.002)) for the residuals and normal distributions centred on zero with large variances as fixed effects priors (default prior in MCMCglmm). This model was run across 1,000,000 iterations with thin of 600 and a burn in of 1,500. Here, the potential scale reduction factor was 1.001 or lower and the autocorrelation was not higher than 0.03 [50]. In this analysis 11 Kentish plovers were excluded from the model because wing length, body mass or both measurements were not available. MCMCglmm results are expressed as posterior mean, lower and upper 95% credibility intervals, and significance as a pMCMC value. All statistical analyses were conducted in R v3.3.3 [54].

Results

Forty-seven out of 219 birds sampled were infected (21.5%). The highest prevalence was recorded in *Salmonella* (15.1%) followed by *Chlamydia* (4.6%) and *Campylobacter* (1.8%), with no birds presenting mixed infections. Bacteria infection was spread out in most populations (Table 1) except on Canary Islands where only *Salmonella* was found (Table 1).

Bacteria prevalence was always higher in birds from mainland than from islands, however this difference was non-significant. Although a trend appeared when infection was pooled together, showing nearly significant higher prevalence of infection in mainland ($P = 0.077$; Fig 2, Table 2). Females had a higher prevalence of *Salmonella* than males, and the same pattern was found when all bacteria were pooled together (Fig 2, Table 2). The interaction between insularity and sex was not significant (all cases $P > 0.05$), so insularity did not affect bacteria prevalences between the sexes.

Body condition was not significantly affected by the presence of the three bacteria (Fig 3, Table 3). Females were heavier than males in the mainland while on islands no sex differences in body condition were found (Table 3).

Discussion

Our results showed that *Campylobacter*, *Chlamydia* and *Salmonella* were widespread among most Kentish plover populations and similarly prevalent in mainland and islands. Female

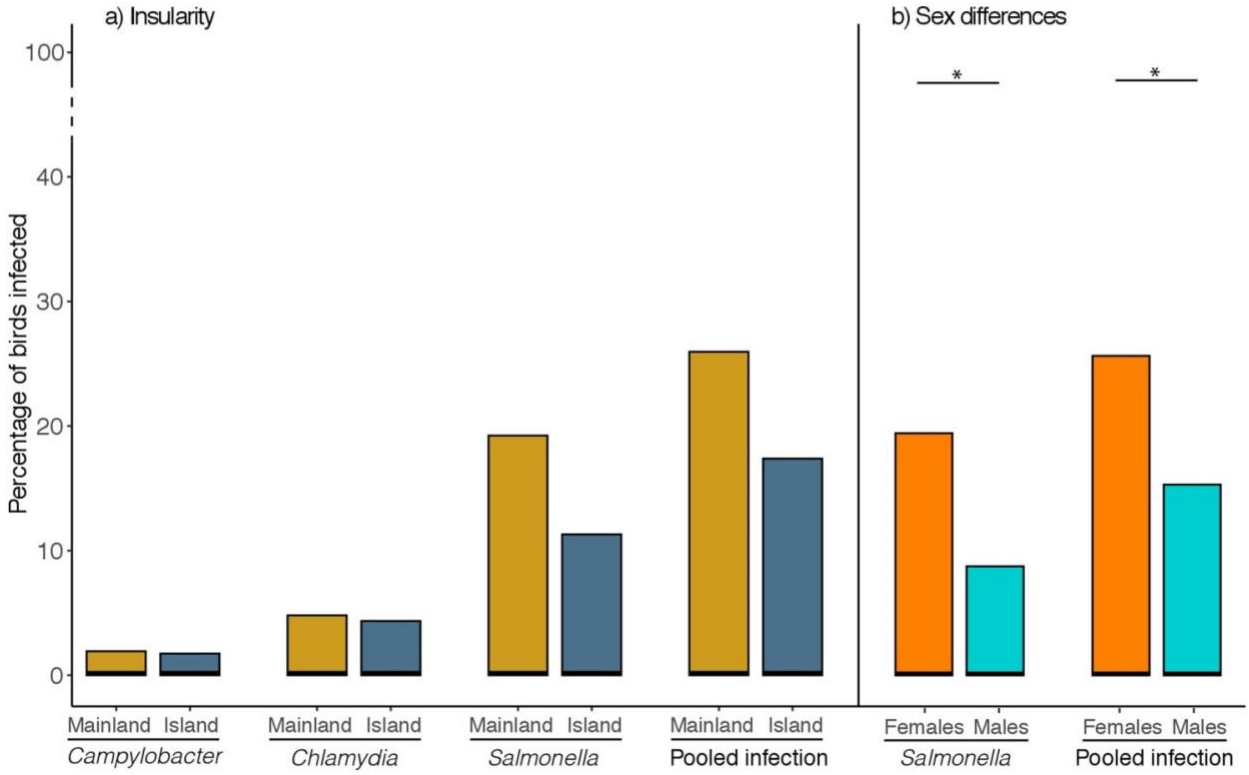

Fig 2. Differences in bacteria prevalence between populations. Prevalence of cloacal bacteria infection between a) mainland and island and b) male and female Kentish plovers. *Indicates a statistically significant difference of $P < 0.05$.

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Kentish plovers had a higher *Salmonella* prevalence than males, a pattern also found when the infection of the three bacteria was combined together. Lastly, we showed that body condition was not related to infection of the three bacteria but to sex and insularity, with a higher body condition found in females and in birds from the continent.

Insularity

We found similar *Campylobacter*, *Chlamydia* and *Salmonella* prevalences in insular and mainland bird populations. One reason of our findings in *Salmonella* could originate from the fact that infection with this genus of bacteria, as most shorebird microbiota, depends on the environmental availability of the bacteria [55]. Although Kentish plovers in our study bred in completely different landscapes (i.e. islands vs mainland), the breeding sites were relatively similar in that involved lands of high salinity, scarce vegetation and close to saline water bodies. High levels of salinity could consistently constrain bacteria acquisition throughout sampling locations because salinity is a well-known inhibitor of *Salmonella* and *Campylobacter* growth [56, 57]. Perhaps the exception to this was the population from Doñana that bred near brackish water. Interestingly, the percentage of infected birds in Doñana was equal to those in Cádiz (19.2%) but higher than in Lanzarote and Maio (respectively, 11.1% and 11.4%). The animal diversity in mainland increases the probability of encountering animals hosting *Salmonella* infection that could later be acquired by Kentish plovers [58], and thus is a possible

Table 2. Infection of (a) *Campylobacter*, (b) *Chlamydia*, (c) *Salmonella* and (d) all bacteria combined in relation to insularity and sex in Kentish plovers (n = 219).

		95% credibility intervals		
	Post. mean	**Lower**	**Upper**	***P***
a) Intercept	-5.196	-7.807	-2.984	< **0.001**
Insularity (island)[a]	0.002	-3.833	3.726	0.995
Sex (males)[b]	0.533	-2.906	4.687	0.754
Insularity (island)[a]*sex (males)[b]	-0.324	-5.604	4.788	0.876
Random				
Site	0.027	0.006	0.063	
Date	0.028	0.007	0.066	
b) Intercept	-3.257	-4.519	-2.139	< **0.001**
Insularity (island)[a]	-0.945	-3.140	1.140	0.360
Sex (males)[b]	-1.434	-4.541	1.275	0.318
Insularity (island)[a]*sex (males)[b]	2.276	-1.385	6.014	0.197
Random				
Site	0.029	0.006	0.071	
Date	0.028	0.006	0.064	
c) Intercept	-1.349	-1.991	-0.630	< **0.001**
Insularity (island)[a]	-0.842	-1.958	0.219	0.129
Sex (males)[b]	-1.331	-2.584	0.094	**0.043**
Insularity (island)[a]*sex (males)[b]	0.520	-1.272	2.644	0.578
Random				
Site	0.027	0.006	0.061	
Date	0.030	0.006	0.076	
d) Intercept	-0.887	-1.53	-0.230	**0.008**
Insularity (island)[a]	-0.920	-1.939	0.110	0.077
Sex (males)[b]	-1.248	-2.526	-0.146	**0.030**
Insularity (island)[a]*sex (males)[b]	0.980	-0.693	2.678	0.252
Random				
Site	0.032	0.006	0.078	
Date	0.030	0.007	0.074	

Residual variances were fixed at 1. Significant effects in bold.

[a]Relative to mainland.

[b]Relative to females.

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reason of the close to significant higher prevalence of *Salmonella* in the continent. However, a possible counter argument is that hot environments (25–35 degrees C) with high relative humidity such as the islands of Maio and Lanzarote, provide suitable conditions for a longer persistence in the environment of the bacteria [59], increasing the potential of between-individuals bacteria transmission through ingestion of bacteria from feathers during preening, posterior to, for example, belly-soaking [more frequent in hot environments, 60] or direct contact of individuals (e.g. copulation) [34]. *Campylobacter* may be also acquired from the environment and thus could be affected by the same factors described for *Salmonella* [61]. However, *Campylobacter* is much more susceptible to environmental conditions, requiring, for example, microaerophilic conditions to proliferate [57, 62]. Another reason of such low prevalences found (1.8%, 4 infected out of 219 birds) could be because direct PCR detection from feaces can be problematic compared to enrichment and culture, regarded as the gold

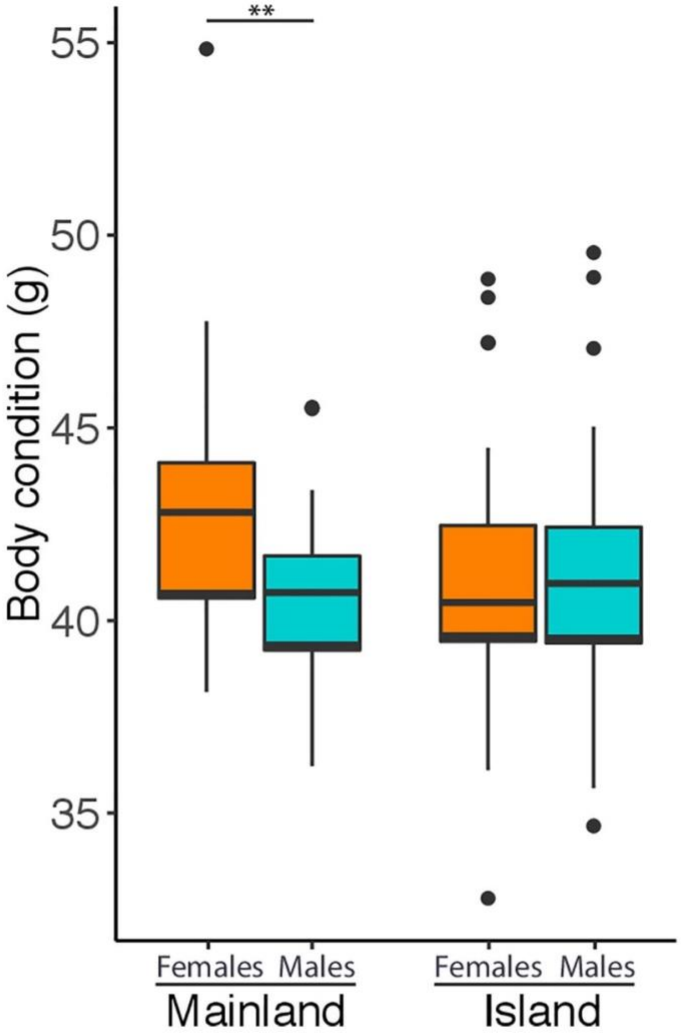

Fig 3. Variation in body condition in Kentish plovers. Scaled mass index in male and female Kentish plovers from mainland and islands (females and males from mainland weighed on average [mean ± standard deviation] 42.6 ± 2.9 and 40.5 ± 2.1 g, respectively, while on islands, females and males weighed 41.0 ± 2.8 and 41.0 ± 3.1 g, respectively). Medians, upper and lower quartiles are shown. Whiskers indicate minimum and maximum values and circles outliers. **Indicates a statistically significant difference of $P < 0.01$.

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standard for *Campylobacter* detection [63]. Although *Campylobacter* prevalences in the wild are medium to high and around 75% in shorebirds [64], a previous study investigating the prevalence of *Campylobacter* spp. in Kentish plovers failed to find any infected individual out of 12 tested [65]. In addition, low prevalences, as found in *Campylobacter* and *Chlamydia*, may make more difficult to detect differences in prevalence between populations. This is particularly important for bacteria like *Chlamydia*, that is mainly horizontally transmitted by direct contact between infected individuals [66].

Table 3. Factors affecting the body condition in Kentish plovers (n = 208).

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
Intercept	**42.656**	**41.802**	**43.486**	**< 0.001**
Pooled bacteria infection	-0.195	-1.578	1.151	0.789
Insularity (island)[a]	-1.815	-2.964	-0.639	**0.001**
Sex (males)[b]	-2.196	-3.445	-1.029	**0.001**
Pooled bacteria infection*Insularity (island)[a]	0.958	-0.888	3.081	0.342
Pooled bacteria infection*sex (males)[b]	0.198	-1.730	2.407	0.841
Insularity (island)[a]*sex (males)[b]	2.236	0.702	3.838	**0.006**
Random				
Site	0.028	0.006	0.071	
Date	0.029	0.006	0.067	
Residual	8.047	6.529	9.587	

Eleven birds were excluded from the model. Significant effects in bold.

[a]Relative to mainland.

[b]Relative to females.

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Sex-specific bacteria prevalence

Bacteria prevalence was significantly female-biased when *Salmonella* infection and all the bacteria species were analysed together. These sex differences were independent of insularity. In addition to potential differences in the ecology of the different bacteria genera, the very low prevalences of *Chlamydia* and *Campylobacter* may explain why our results only approached significance for *Salmonella*. Studies of sex-specific parasite infection (as general term) have shown great heterogeneity in their patterns and are rather scarce in terms of bacteria presence. One study investigating pathogen prevalence in the island populations of Berthelot's pipit (*Anthus berthelotii*) found no sex differences of infection with pox virus, *Plasmodium* and *Leucocytozoon* [67]. Another study found that females had higher prevalence of cloacal bacteria than males in alpine accentor (*Prunella collaris*) [68], while bacteria richness did not vary with sex in blue tits (*Cyanistes caeruleus*) [69]. This heterogeneity may be due to many non-exclusive factors, including differences in immunocompetence and behavior between host sexes as well as differences in the ways of transmission between the pathogens studied [70–72]. The immune system plays an important role in pathogen defense hence if sex-specific differences in immunocompetence exist, we could expect unbalanced infection. However, although a recent meta-analysis showed in general no sex differences in immune capacity across animals [including birds, 73], the literature available shows plenty of variation (i.e. female and male biases) at species and population level that has not yet been explained [74–77].

Body condition

Recent advances in methods of microorganism detection have shown that wild animals often are natural reservoir of pathogenic microorganisms without any apparent health cost [38, 78, 79]. The relationship between bacteria infection and body condition could be difficult to untangle because one could argue that individuals in poor body condition would be more prone to infection, however, this is more likely to happen when access to food is reduced and immunity also gets compromised [80, 81]. Nevertheless, the consistent presence of these bacteria that we found throughout the locations and populations of Kentish plover, in addition to the lack of impact on body condition, suggests that these shorebirds are natural reservoir of

Campylobacter, *Chlamydia* and *Salmonella*. However, these bacteria have great diversity of strains with different pathogenicity and the impact of parasites on host health, survival and life history is difficult to demonstrate based on observational studies and always require experimental manipulation of parasite prevalence or intensity of infection [82, 83]. Further studies are needed to determine whether positive birds harboured strains distinctive and specific to Kentish plover, or strains of other species that have recently adapted to this host [28]. Contrary to our expectations, birds from mainland had better body condition than those from islands. Animals living on islands are exposed to low interspecific competition for food [84]. In addition, the tropics lack of well-defined seasons, with rather stable temperatures during the day and night, and predictable foraging conditions. Such conditions could prevent birds from fuelling up excessively and store energy as fat because of the constant food availability. Also, animal in tropics tend to have slower basal metabolic rates, which imply lower caloric requirements [85]. On the other hand, birds from mainland are exposed to more variable environmental condition like lower temperatures at night, that might translate into higher food consumption during the day [86]. Interestingly, only birds from mainland had sex differences in body condition. Sex differences in body mass or body condition during breeding usually occur previous to the egg-laying stage, where females increase their body mass, and then later during the nesting and brood care stage, where the sex that provides most of the care will see the detrimental effects on body weight [e.g. 87–89]. If we take this into consideration, in addition to previous studies in different continental Kentish plover populations describing a polyandrous mating system [90], we could argue that a polyandrous mating system could explain why only in mainland we found that females were heavier than males, because in polyandry males provide the brood case. However, this postulate cannot be confirmed because to date, no empiric studies have investigated the mating system of the mainland populations here studied.

Conclusion

Although in a relatively low prevalence, our study shows that *Campylobacter*, *Chlamydia* and *Salmonella* were widely present across Kentish plover populations, placing it as possible natural reservoirs of these bacteria. Contrary to our expectations, the three bacteria examined were equally prevalent on mainland and on island populations. Insularity and the sex of the host were important variables determining the bird's body condition, but these patterns were difficult to interpret. Positive relationships between geographical size and animal, plant and bacteria diversity have been reported [e.g. 9, 91]. In the case reported here, it is possible that bacteria infection in hosts do not directly depend on geographical size because of the added level of complexity of including the many variables of the host that could also be affected by insularity. We emphasize on expanding research on bacteria infection in wild birds from an ecological point of view, necessary to further understand the potential impact of social interactions and mating system structure on sexual differences in the prevalence of cloacal bacteria.

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Chapter 3 | Mortality cost of sex-specific parasitism in wild bird populations

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Mortality cost of sex-specific parasitism in wild bird populations

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Sex-specific mortality is frequent in animals although the causes of different male versus female mortalities remain poorly understood. Parasitism is ubiquitous in nature with widespread detrimental effects to hosts, making parasitism a likely cause of sex-specific mortalities. Using sex-specific blood and gastrointestinal parasite prevalence from 96 and 54 avian host species, respectively, we test the implications of parasites for annual mortality in wild bird populations using phylogenetic comparative methods. First, we show that parasite prevalence is not different between adult males and females, although Nematodes showed a statistically significant but small male-biased parasite prevalence. Second, we found no correlation between sex-biased host mortalities and sex-biased parasite prevalence. These results were consistent in both blood and gastrointestinal parasites. Taken together, our results show little evidence for sex-dependent parasite prevalence in adults in wild bird populations, and suggest that parasite prevalence is an unlikely predictor of sex difference in adult mortalities, not withstanding sampling limitations. We propose that to understand causes of sex-biased mortalities, more complex analyses are needed that incorporate various ecological and life history components of animals life that may include sex differences in exposure to predators, immune capacity and cost of reproduction.

Although sex ratio at birth is often close to 1:1 in wild populations, adult (or tertiary) sex ratios (the proportion of males to females in a population) are highly variable suggesting that sex differences in post-birth maturation, mortalities and/or population movements drive skewed adult sex ratios (ASR)[1–4].

Mortality is a complex process, influenced by many factors that in simple terms could be classified as intrinsic and extrinsic to the individual (for example, immune capacity and ambient environment, respectively[5]). Predation, disease and starvation are important causes of mortality in wild animal populations, whereas body size and sexual selection are general predictors of mortality according to life history theory[6,7], with larger animals often dying at lower rates than smaller ones[8–11]. Furthermore, social activities such as competition for food and/or mates may increase mortality of one sex more than the other[12–14].

One important cause of mortality are pathogens or infectious agents. For instance, the history of the modern human has been marked by diseases of epidemic scale that resulted in millions of deaths that were caused by bacteria, viruses and parasites[15]. Most recently, the COVID-19 pandemic, although with relatively low mortality, showed to be more lethal for men than for women[16]. In wild animals, examples of elevated mortality due to pathogen infection often include native species exposed to exotic pathogens, driving populations to critically low numbers (e.g. Darwin's finches[17], Serengeti's wild dogs and lions[18]) or even to the edge of extinction[19]. Moreover, pathogens have shown to also provoke mortality not by directly killing the host but debilitating and deteriorating their overall condition, increasing the chances of predation[20–23].

Interestingly, despite the presumed relationship between pathogens (i.e. biological agent that causes disease or illness) and mortality in animals, information on the relationship between sex-biased infections and biased sex ratio is scarce. A notable exception occurred in mammals, where Moore and Wilson[1] found a positive correlation across 106 mammal species for the bias in sexual size dimorphism (SSD) and the sex bias in parasitism, and that sex bias in parasitism predicted the sex bias in mortality, concluding that sexual selection for the larger sex (i.e. males) implicated a mortality cost through parasitism (see also[24]). Also, male mammals have a weaker immune competence, which correlates with higher presence of pathogens and mortality compared to females[25,26]. In birds, sex-biased infections and its implications on survival have not been assessed across a broad

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range of taxa, although indirect evidence would suggest so since a previous across-species meta-analysis found a male-biased prevalence of gastrointestinal parasites[27]. In addition, more recent complementary evidence shows that larger avian species are more affected by parasites, possibly because in larger hosts, parasites have greater space and niches to colonize and are likely to accumulate through life as larger species tend to live longer than smaller ones[9,28,29]. Therefore, from this perspective, and considering that in birds males are in general larger than females[30], we could expect parasitism in birds to be male-biased. Although the difference in body size of birds is modest compared to mammals, it is unknown at what extent this could influence parasite load between the sexes. From a hormonal perspective, the sex hormones influence the reproductive behavior e.g. courting, territoriality, aggression, competition and nesting[31,32], which could translate into sex-different rates of parasite acquisition due to immunosuppression mediated by testosterone or stress-released corticosterone (cost for males[25,33,34]), or sex-differences in behavior such as nesting time or breeding dispersal (cost for females[35,36]). However, current studies disregard the effect of sex hormones in immunity, as well as challenge the idea of sex-different immune response in birds[37,38], finding little evidence supporting a significant effect of sex hormones in immunity in studies using physiological concentrations of hormones. Moreover, another study[26] showed a lack of sex-differences in immunity across 241 immune estimates in birds, while recent evidence showed that, in general, immunosenescence also lacked sexual dimorphism across animals, including birds[39].

Thus, the current evidence highlights males but not females as the sex more likely to be affected by parasites in birds[27,28,34], although it is nevertheless unknown whether this variable could relate to the overall lower male mortality compared to females found in birds[13], and suggested by their overall male-biased ASR[3,40].

Nevertheless, studies using unsexed birds suggest an association between mortality and both blood and gastrointestinal parasitism, supported by evidence established through direct analysis of carcasses of mortality events or through capture-recapture survival analyses[41–44]. Moreover, blood (protozoan and microphilaria) and gastrointestinal parasites (helminths and coccidia) have different means of transmission that in turn could also influence patterns of sex-specific infection and thus mortality. For instance, nest type (open versus close) is often considered a risk factor for malaria infection because open-nesting offers increase exposure to dipteran vectors such as mosquitoes[45].

To examine the relationship between sex-specific parasite prevalence and mortality, we obtained data from a total of 138 bird species (across 96 species from 13 avian orders for blood parasites and 54 species from 9 orders for gastrointestinal parasites) from published literature to test two hypotheses using phylogenetic comparative analyses. We use parasite prevalence because it gives an estimation of the infection status of a population, thus providing hints of their susceptibility to parasite infection (although not without limitations[46]). Also, determinants of parasite prevalence depend on a number of ecological and behavioral variables[47] that could differ between the sexes[48], as well as being one of the most commonly available parasite estimates in parasitology and ecology. First, we investigate whether males had higher parasite prevalence compared to females, as predicted by male's modest but significantly larger body size[30], male's frequent stress-inducing behavior (corticosterone mediated immunosuppression[32,34]), and as previously shown in across-species studies in mammals[1] and in birds (particularly gastrointestinal parasites[27]). Second, we evaluate whether sex-specific parasite prevalence predicted sex-specific adult annual mortality. Specifically, we (i) test the effect of parasite prevalence on mortality in males and females separately as they present variation in their physiology and life histories[49] that could influence the degree of exposure and/or infection to parasites and subsequent mortality[48,50]. Finally, we (ii) evaluate whether sex-specific adult annual mortality is predicted by sex-specific adult parasite prevalence, including SSD and mating competition in the analysis as potential confounding variables[24,51,52].

Material and methods

Literature search. We collected data on sex-specific prevalence of parasitism in birds using ISI Web of Science and Google Scholar. The use of Google Scholar in systematic reviews has been recently criticized[53], however, in our study we used Google Scholar because it expands searches to include grey literature, such as technical reports and theses. The searches were conducted by using the following keyword combinations: "scientific name of host species" + parasit*, prevale*, helmint*, blood, malar*, haemoparasit*, mite* or lice. Because our aim was to evaluate the effect of parasitism on sex-specific mortality, the list of names searched initially corresponded to 369 bird species included in the dataset of sex-specific annual mortality data provided by Székely et al.[52]. If the bird species name had synonyms, the search was repeated with every name. The references of previous reviews and meta-analyses were also checked (see supplementary material). The inclusion criteria required the parasite prevalence to be: (i) determined from adult birds with known sex, (ii) obtained from wild birds (not captive), and (iii) from infection naturally acquired (not experimentally infected). We only included studies reporting results for both males and females to avoid difficulties comparing prevalences within species generated by different sampling/diagnostic methods or different populations. We included studies with haemoparasite detection through molecular and optic microscopy methods because both bring comparable results and to date there is not consensus about which technique is better over the other[54,55]. All studies available for gastrointestinal and external parasites used exclusively taxonomic keys diagnosis through microscopic examination. Studies based on parasite's egg counts were not considered to minimize the chances of including studies containing false negative results originated by the variation in egg shedding rhythms seen in some gastrointestinal parasites[56]. In order to obtain a robust estimate of parasite prevalence for a given host species, all publications that met the inclusion criteria were included in our dataset. Further details of the literature search as well as the full list of studies consulted are given in the supplementary material (Tables S1 and S2).

Body mass, adult mortality and sexual competition. Data on sex-specific body mass, annual adult mortality and sexual competition were obtained from Székely et al.[52]. Data were augmented following the

method provided by Székely et al.[52] and Liker et al.[57], consisting of searching the name of the additional bird species in scientific citation indexes, books, species monographs and electronic databases (see supplementary material). We included mortality estimates obtained from field studies in which the estimates for both males and females were determined in the same population and with the same method. Three main methods were used to determine mortality rates: capture-recapture, ringing recoveries and local return rates. Mating system was determined as a five-point score by the frequency of polygamy for each sex, with "0" corresponding to very rare or no polygamy, "1" to rare polygamy, "2" to uncommon polygamy, "3" to moderate polygamy and "4" to common polygamy (for more details see[57]).

Parasite prevalence. The final dataset included 96 bird species (sample size range of 4–1045) with sex-specific blood parasite prevalence data, 54 species (5–9729) with gastrointestinal parasite prevalence data and only 3 species (13–131) with ectoparasite prevalence data. Ectoparasites were excluded from further analyses due to the low sample size. Blood parasites were divided into five categories: *Haemoproteus*, *Leucocytozoon*, *Plasmodium*, *Trypanosoma* and Microfilaria. Whereas gastrointestinal parasites were categorized as Cestoda, Acanthocephala, Nematoda, Trematoda and Protozoa. Finally, one last category received data presented as the combination of two or more parasite categories (for example, we often found blood parasite studies reporting the overall prevalence of *Haemoproteus*, *Leucocytozoon* and *Plasmodium*, three parasites categories combined in one single datum). Some studies of blood parasitism included avian species that presented 0% prevalence in both sexes. These studies were included in the dataset, although it was uncertain whether birds never got parasitized due to vector absence in their habitats[58], were able to reduce parasitemia under detection limits, or because the parasites were unable to complete their life-cycle in the host[59]. Along with parasite prevalence data we also recorded the period of the year when parasites were samples, which was divided in three categories: breeding (sampling took place mostly during the hosts' breeding period), nonbreeding (sampling took place outside the breeding period) and year-round (sampling included both breeding and nonbreeding periods).

Phylogenetic meta-analysis. To investigate sex difference in parasite prevalence, a phylogenetic multilevel meta-analysis was performed using the R package *metafor*[60]. Because all studies only provided prevalence and sample size values, we opted to group the birds as infected and not infected males and females in 2×2 contingency tables and then calculate the effect size as log odds ratio[60]. We conducted the meta-analyses including period of sample (breeding, nonbreeding and year-round) and method of parasite detection (only for blood parasites, consisting of three categories: molecular detection, optic microscopy detection, and both) as moderators (i.e. fixed-effect), and study and phylogeny (a variance–covariance matrix) as random-effect variables.

Publication bias (due to missing studies that were not published because of negative or null results[61]) was evaluated using Egger's regression test[62,63] by including the standard error of the effect sizes as an additional moderator within the model. If the intercept significantly deviated from zero (significance of $P < 0.10$[62]) the overall relationship between the precision and size of studies included in the dataset was considered asymmetrical, or in other words, biased[63]. Of the twelve models conducted, two suggested presence of publication bias, corresponding to the gastrointestinal parasites Nematoda ($P = 0.035$) and Trematoda ($P = 0.043$). Diagnostic tests for identifying influential data points and outliers, and rules for excluding these types of cases are still evolving, particularly for multivariate/multilevel meta-analytical models[64]. To address this, our approach consisted of identifying the influential outliers causing the bias and running the models after excluding these values [see[65]].

Statistical power in random-effects meta-analysis can be difficult to determine. It has been suggested that, in general, meta-analyses with at least five studies offer more power than the individual studies alone[66]. Therefore, outcomes below this five-studies threshold should be taken carefully.

Phylogenetic comparative analysis. We used phylogenetic generalized least squares (PGLS) to test whether parasite prevalence was related to annual mortality, adult body mass and sexual competition. This approach allows controlling for the non-independence among species by incorporating a variance–covariance matrix that represents their phylogenetic relatedness[67]. In all models we used Pagel's lambda (λ) as measure of phylogenetic signal[68] and it was set to the maximum-likelihood value[69]. Prior to the analyses, prevalence and mortality were logit-transformed[70]. Mortality bias was expressed as log(male mortality/female mortality). Average body mass (in grams) of male and female adults was log-transformed, whereas SSD in adult body mass was expressed as log(male body mass (g)/female body mass (g)). The sex bias in mating system was calculated as the difference between male and female polygamy scores[57]. Because often each host species had several estimates of prevalence (i.e. studies reporting estimates for more than one parasite group), the sex bias in parasite prevalence of each bird species was incorporated into this analysis as the weighted average effect size of all comparisons. Instances where multiple studies reported prevalence estimates for the same host species were handled by adding sister tip labels (of the same branch length) to the phylogeny. The effect size per species was calculated using the function *escalc* of the R package *metafor* with log odds ratio as measure. We fitted both single-predictor and multi-predictor models to blood parasites and gastrointestinal parasites and each model was run separately for females, males and sex bias. To account for phylogeny, we used the avian phylogeny from Jetz et al.[71]. The analyses were run using consensus trees (one for each type of parasitism, Fig. S1) obtained through the method 50% majority-rule[72,73] from 1,000 randomly selected trees from a pool of 10,000 available (https://birdtree.org), using the methodology described by Rubolini et al.[74]. These phylogenetic trees were not fully resolved, and polytomies were arbitrarily resolved by adding a branch distance of 10^{-08} to one randomly chosen branch in the polytomy using the function *multi2di* from the R package *ape*[75]. All PGLS analyses were conducted in R using the package *caper*[76].

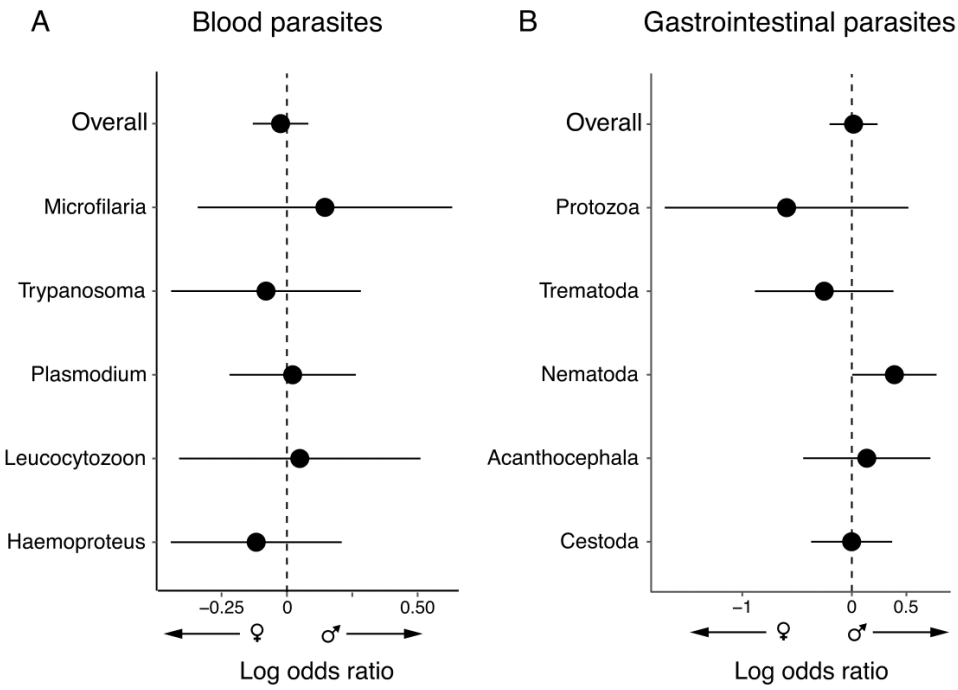

Figure 1. Sex bias in prevalence of (**A**) blood parasites and (**B**) gastrointestinal parasites in birds. Weighted average effect size estimates, showing lower and upper 95% confidence intervals in overall meta-analyses and broken down results according to parasite category (see Material and methods). The dashed vertical line indicates no sex difference, positive values represent male bias prevalence and negative values female bias. See Table 1 for statistics.

Results

Phylogenetic meta-analysis. Overall, males and females did not exhibit different prevalence of blood parasites nor gastrointestinal parasites (Fig. 1 and Table 1). In the analysis broken down for parasite category (five categories of blood parasites and five of gastrointestinal parasites; Table 1), only Nematodes showed a weak male-biased prevalence (Fig. 1b; $k = 33$, estimate = 0.388, Z statistic = 1.979, $P = 0.048$, 95% CI = 0.004, 0.773).

Parasite prevalence and annual adult mortality. We found no association between annual mortality and prevalence in either blood parasites or gastrointestinal parasites (Table 2). The lack of association was consistent when each sex was tested separately (Table 2) and also when analyzing the sex bias (Fig. 2 and Table 2).

These results remained qualitatively unchanged after conducting multi-predictor analyses incorporating body mass and mating competition into the models (Table 2). In these latter analyses only body mass and mating competition had a significant effect on mortality, although the relationship with mating competition was significant only in the blood parasite analyses (Table 2a).

In most cases the phylogenetic signal (λ) was moderate to high, indicating important variation associated to phylogenetic relatedness, however, further examination considering avian orders show no clear clustering for sex bias analyses (Fig. 2 and S2).

Discussion

To our knowledge, this work represents the largest comparative study of sex-specific parasite prevalence in birds, based on 96 species with sex-specific blood parasite prevalence data and 54 species with gastrointestinal parasite prevalence data. Taken together, our results showed little evidence supporting sex biases in parasite prevalence, with no overall sex bias in blood or gastrointestinal parasites prevalence in birds. Additionally, no relationship was found between sex bias in mortality and sex bias in parasite prevalence, even after controlling for possible confounding life history variables, i.e. mating system, body size and sexual size dimorphism.

Our findings do not support the prediction of male-biased parasitism generated by the sexual size dimorphism[1,30] and sex-different hormonal immunosuppression[31,33]. One possible explanation is that in birds the magnitude of the difference in size between sexes tends to be smaller compared to mammals[77], where an association between sexual size dimorphism and parasite prevalence has been shown[1]. Furthermore, some evidence shows little and no relationship between body size and blood parasites across avian species[47,78,79]. Scheuerlein and Ricklefs[80] found an association in parasite prevalence and body size in passerines, however, after controlling for phylogeny, the association was marginal. On the other hand, although stress and sex hormones were not part of

	Q_{REML} (*P*-value)	*k*	*n*	Studies	Estimate (95% CI)	*Z* statistic (*P*-value)
Prevalence of blood parasite (overall)	265.994 (0.043)	229	96	78	−0.024 (−0.130, 0.082)	−0.451 (0.652)
Haemoproteus	61.240 (0.575)	69	60	51	−0.117 (−0.444, 0.210)	−0.704 (0.481)
Leucocytozoon	39.710 (0.6559)	49	43	33	0.049 (−0.413, 0.511)	0.209 (0.835)
Plasmodium	30.820 (0.822)	44	39	29	0.022 (−0.220, 0.263)	0.178 (0.859)
Trypanosoma	17.257 (0.8375)	28	23	21	−0.080 (−0.443, 0.283)	0.186 (0.666)
Microfilaria	5.186 (0.878)	13	10	10	0.145 (−0.341, 0.632)	0.591 (0.555)
Prevalence of gastrointestinal parasites (overall)	226.818 (<0.001)	116	49	37	0.016 (−0.203, 0.234)	0.140 (0.889)
Cestoda	68.354 (<0.001)	27	23	22	−0.002 (−0.372, 0.368)	−0.011 (0.991)
Acanthocephala	6.141 (0.726)	12	10	10	0.137 (−0.444, 0.717)	0.461 (0.645)
Nematoda	37.544 (0.162)	33	22	20	0.388 (0.004, 0.773)	3.918 (0.048)
Trematoda	20.086 (0.389)	21	11	8	−0.252 (−0.885, 0.380)	−0.782 (0.434)
Protozoa	12.537 (0.484)	15	15	5	−0.596 (−1.708, 0.516)	−1.050 (0.294)

Table 1. Phylogenetic meta-analysis of sex difference in prevalence of blood parasites and gastrointestinal parasites. The estimate represents the weighted average effect size as log odds ratio and its positive or negative value represents the sex bias directionality (see Fig. 1). Meta-analyses were performed using multilevel random-effect meta-analysis with restricted maximum likelihood (REML). Fixed-effect variables: period of sampling and method of parasite detection. Random-effect variables: phylogenetic relatedness and study. Q_{REML} = test for heterogeneity; *k* = number of effect sizes; *n* = number of host species; Studies = number of studies.

our analysis, our results give little support to the idea of sex-differences in corticosterone immunosuppression, and seem to be in line of with recent research finding inconclusive results in the immunocompetence handicap theory in birds[26,37,81].

Specifically, we found no sex bias in the overall prevalence of blood parasites, consistent with the overall results of a previous meta-analysis of blood parasites in birds[79]. Sex differences in blood parasites are generally thought to occur due to unequal exposure of the sexes to vectors[82,83] and differences between males and females in the immune-endocrine system[84]. Perhaps the lack of sex differences seen here could be attributed to these processes balancing each other out. For example, in males, the persistent pressure of male-male competition could generate stress-induced corticosterone which due to its immunodepressive effect could make them more prone to infection[34], at the same time that the elevated exposure of females to vectors while incubating[35]. Poulin[27] found a strong male-biased infection of Acanthocephalan and Nematodes parasites, consistent with our results in the overall parasite prevalence in Nematoda. Nematodes are a very diverse group of round worms. Male-biased parasite prevalence in this group could be due to many non-exclusive variables including those previously suggested for overall gastrointestinal parasites (mainly based on differences in body size; see Introduction), in addition to sex-specific foraging behavior as result of niche specialization or competitive exclusion by the dominant sex[85,86]. However, more studies are needed to test these hypotheses.

Mortality was not related to parasite prevalence across all analyses conducted, even in multi-predictor analyses where mortality was tested against parasite prevalence, body mass and mating system. Only body mass was consistently associated with mortality as found in previous studies[13,52,87]. Although parasite burden has often been linked to mortality in species-specific studies in birds[23,44,88] (but see[89]), here we found that such association seems to be less clear at interspecific level. Nevertheless, our results should be treated cautiously because in most cases parasitism and mortality data did not come from the same population, and because parasite data for males and females is more likely to be reported in studies investigating sexually dimorphic birds, therefore, we cannot discard a possible bias toward sexually dimorphic species over monomorphic ones. In addition, prevalence, as an index of parasitism, could be problematic because it informs about the proportion of infected individuals in relation to the number examined[90], generating uncertainty whether the individuals found positive only correspond to infection-resistant animals that survived the infection[82]. For example, a previous study found that males had lower survival than females to influenza A virus infection[91], therefore, in the hypothetical situation of sampling this population in the wild without knowing this sex-different viral susceptibility beforehand, and assuming a similar infection rate between sexes, females would have a higher prevalence than males because a larger proportion of infected males died.

In contrast to the findings of Moore and Wilson[1] in mammals, sex-biased parasitism in birds did not seem to be a consistent driver of sex-specific mortality. The pressure that parasites impose on birds not only appeared to be low between sexes but also within sexes as no increase nor diminution of mortality were seen when tested males and females separately. Perhaps, juveniles should be the target by further studies to obtain a thorough understanding of mortality patterns. Accordingly, a recent study suggests that juvenile mortality rather than chick and adult mortality corresponded to the main contributor of sex biases in ASR in six plover populations (*Charadrius*)[92]. Unfortunately, juvenile sex-specific parasitism data in birds is scant.

Response variable	Explanatory variable	Slope	*P*
(a) Blood parasites			
Single-predictor models (n = 63)			
Male annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.805$	Male overall blood parasite prevalence	0.009	0.899
Female annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.735$	Female overall blood parasite prevalence	0.039	0.560
Sex bias in annual mortality Adjusted $R^2 = 0.02$; $\lambda = < 0.001$	Sex bias in blood parasite prevalence	0.064	0.167
Multi-predictor models			
Male annual mortality (n = 56) Adjusted $R^2 = 0.18$; $\lambda = 0.994$	Male overall blood parasite prevalence	0.021	0.554
	Male body mass	− 0.208	0.002
	Male mating system	0.109	0.025
Female annual mortality (n = 55) Adjusted $R^2 = 0.18$; $\lambda = 0.925$	Female overall blood parasite prevalence	0.018	0.718
	Female body mass	− 0.242	0.007
	Female mating system	0.252	0.045
Sex bias in annual mortality (n = 55) Adjusted $R^2 = 0.03$; $\lambda = < 0.001$	Sex bias in blood parasite prevalence	0.046	0.223
	Sexual size dimorphism	0.154	0.159
	Sex bias in mating system	0.029	0.056
(b) Gastrointestinal parasites			
Single-predictor models (n = 43)			
Male annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.917$	Male overall gastrointestinal parasite prevalence	− 0.008	0.889
Female annual mortality Adjusted $R^2 = 0.02$; $\lambda = 0.999$	Female overall gastrointestinal parasite prevalence	0.055	0.173
Sex bias in annual mortality Adjusted $R^2 = 0.00$; $\lambda = 0.384$	Sex bias in gastrointestinal parasite prevalence	0.034	0.414
Multi-predictor models (n = 43)			
Male annual mortality Adjusted $R^2 = 0.31$; $\lambda = 0.900$	Male overall gastrointestinal parasite prevalence	0.013	0.791
	Male body mass	− 0.415	< 0.001
	Male mating system	0.138	0.130
Female annual mortality Adjusted $R^2 = 0.170$; $\lambda = 0.950$	Female overall gastrointestinal parasite prevalence	0.005	0.913
	Female body mass	− 0.353	0.005
	Female mating system	0.107	0.435
Sex bias in annual mortality Adjusted $R^2 = 0.44$; $\lambda = 0.999$	Sex bias in gastrointestinal parasite prevalence	0.007	0.749
	Sexual size dimorphism	− 0.936	< 0.001
	Sex bias in social mating system	− 0.009	0.779

Table 2. Phylogenetic generalized least squares (PGLS) showing single-predictor and multi-predictor relationships between annual mortality and prevalence of (a) blood parasites and (b) gastrointestinal parasites. Multi-predictor models include two additional life history variables: body mass and mating system. First each sex was analyzed separately, then we tested the relationship between sex bias in the response and predictor variables (see Material and methods).

In conclusion, our analyses showed that birds do not exhibit overall sexual difference in parasite prevalence, and parasite prevalence do not predict sex-specific mortality, thus suggesting that other processes may drive the sex-differences in adult mortalities reported from numerous bird species. Though, perhaps the limitations in our analysis (mentioned above) contributed to this lack of association. Although life history traits (e.g. mating system, parental care, and body mass) have been shown as important predictors of mortality in birds[13,52,87], the actual etiology that originates female-biased mortality in birds is still poorly explored. Perhaps mortality events during migration[93], predation[94], susceptibility to stress[95], or simply resilience to starvation are more important determining sex-specific mortality than parasites. In addition to this, understanding male versus female immune systems undoubtedly is highly relevant. We call for further comparative and single-species studies to understand the causes of sex different mortality patterns.

Data availability

The full list of references consulted to extract the parasite data is given in the supplementary material. The dataset and R code can be accessed on https://doi.org/10.6084/m9.figshare.13232435.v1.

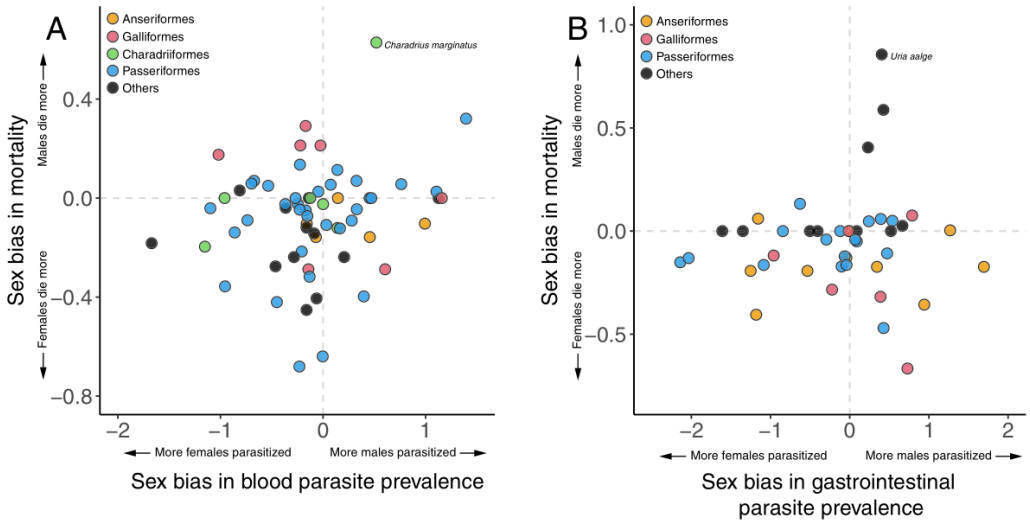

Figure 2. Sex bias in annual mortality in relation to the sex bias in prevalence of (**A**) blood and (**B**) gastrointestinal parasites (see Table 2 for statistics). Sex bias in mortality was expressed as log(male mortality/female mortality), whereas the sex bias in parasite prevalence was expressed as the weighted average effect size of all comparisons (see Material and methods). Represented in colors are the avian orders with the greatest numbers of species in each of the analyses (full species list in supplementary material). Outliers are specified. Dashed lines indicate no sex difference, positive values represent male bias and negative values female bias.

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Author contributions

J.O.V. and T.S. conceived and designed the research; J.O.V. conducted the data analysis and wrote the paper; A.L. and T.S. provided part of the mortality and life history data; all authors contributed substantially to revisions of the paper and gave final approval for publication.

Competing interests

The authors declare no competing interests.

Additional information

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3.1 Supplementary material

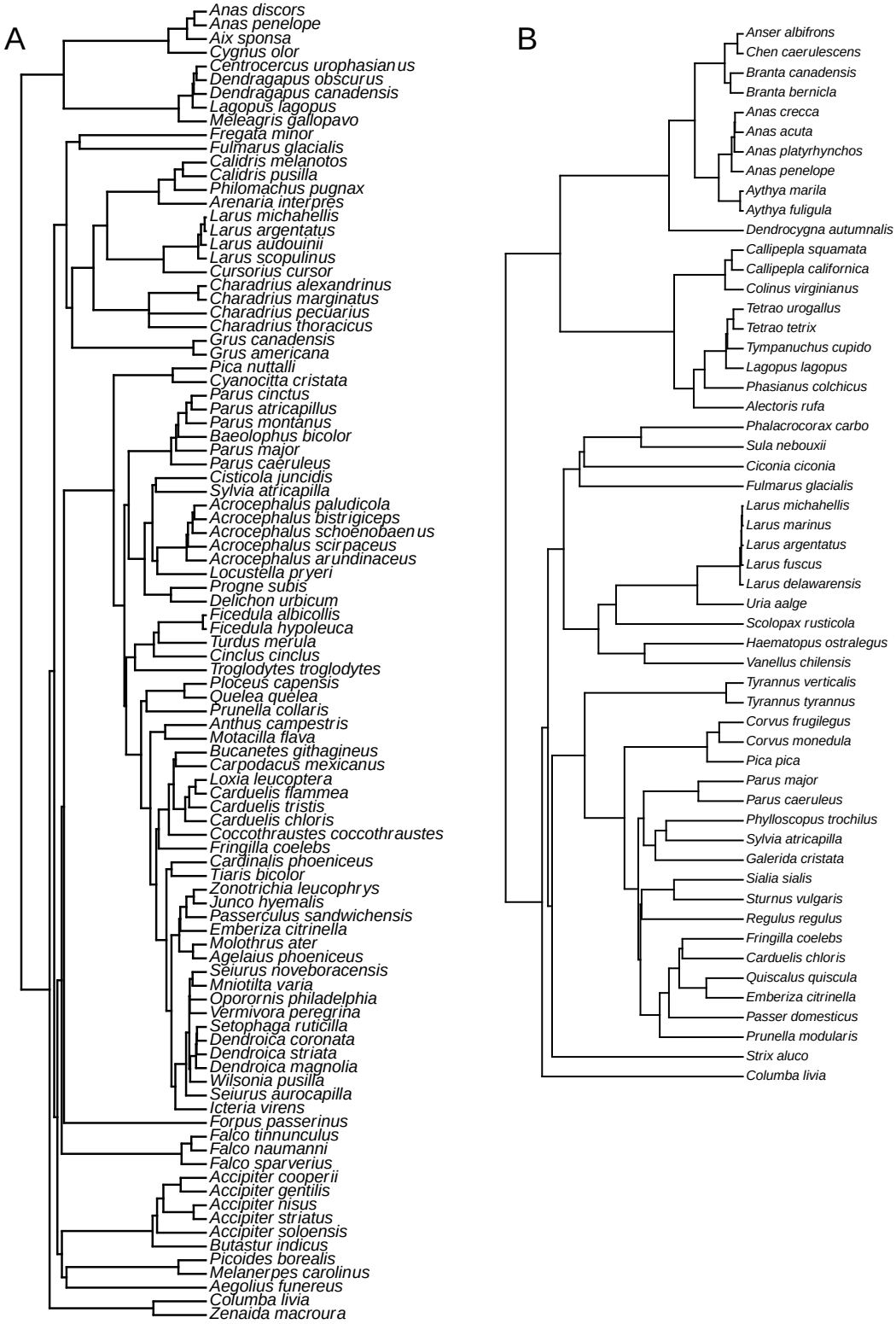

Figure S1. Phylogenetic hypothesis used in the comparative analysis for (A) blood parasites and (B) gastrointestinal parasites.

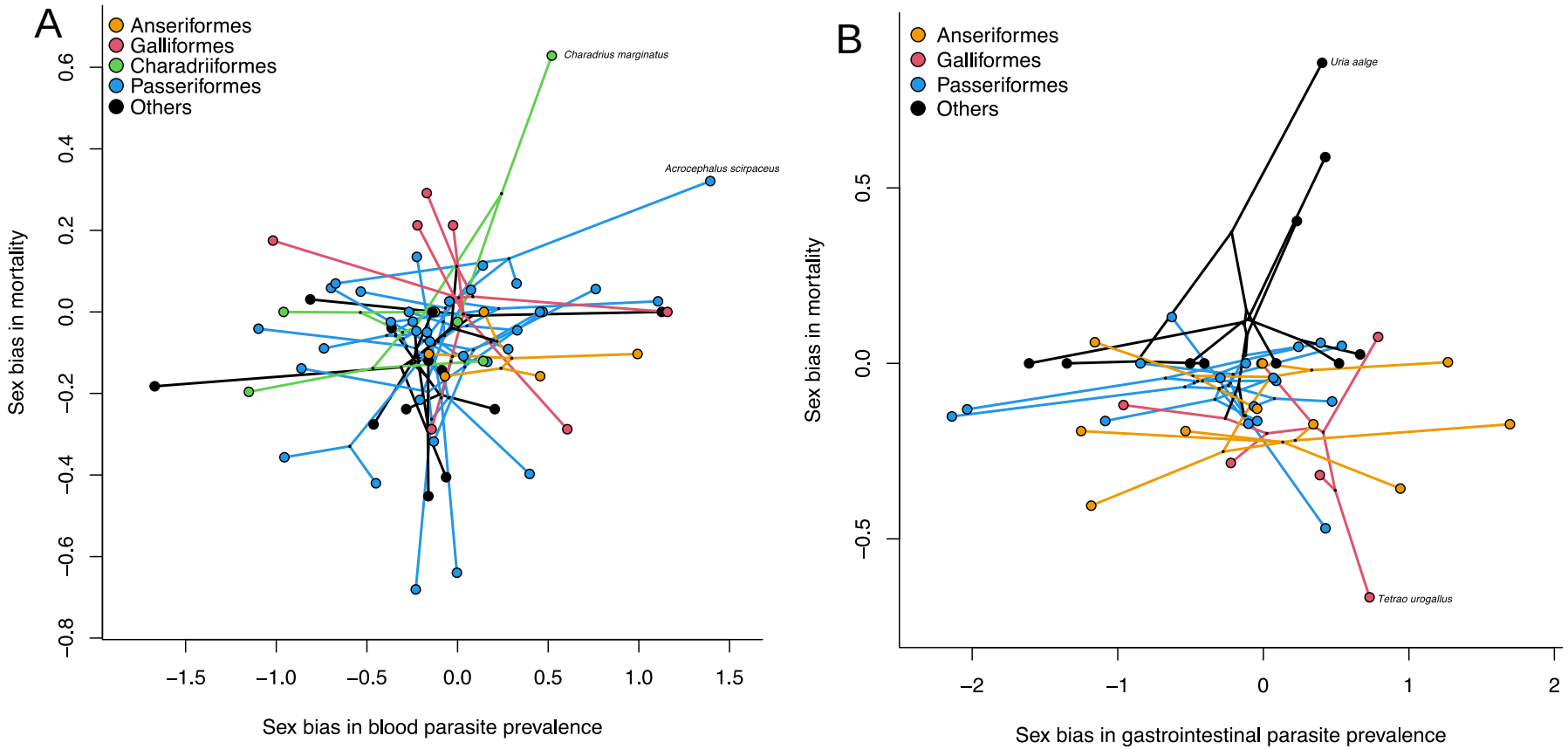

Figure S2. Phylomorphospace plots of the association between the sex bias in annual mortality and (A) the sex bias in prevalence of blood parasites, and (B) the sex bias in gastrointestinal parasites. Represented in colors are the four and three avian orders with the greatest numbers of species in each of the analysis.

Literature search

Because our aim was to evaluate the effect of parasitism on sex-specific mortality, the list of names searched initially corresponded to 369 bird species included in the dataset of sex-specific annual mortality data provided by(Székely et al., 2014) Székely et al. (Proc. R. Soc. B 2014; 281:20140342).

We found 78 studies that met the inclusion criteria for blood parasites and 39 for gastrointestinal parasites. These studies were result of searches on ISI Web of Science, Google Scholar, and consulting the material used in Poulin (Am. Nat. 1996; 147(2):287-295), McCurdy et al. (Oikos 1998; 82(2):303-312). Two studies corresponded to Master of Science theses and one to a Doctoral thesis. Details of the species and each corresponding study used in the analysis are given in Table S1 and S2.

After the initial search, we found blood parasite data for 96 bird species, 38 of which were not listed in Székely et al. (Proc. R. Soc. B 2014; 281:20140342). For these 38 species we collected mortality, mating system and body size data using the methodologies described in Székely et al. (Proc. R. Soc. B 2014; 281:20140342) and Liker et al. (Curr. Biol. 2014; 24(8):880-884). More details are given in the methods section, main text.

For gastrointestinal parasites, from the 54 species found, 7 were not included in Székely et al. (Proc. R. Soc. B 2014; 281:20140342). For these 7 species, data on mortality, mating system and body size was collected as described above.

Table S1. List of 78 studies consulted to extract data on blood parasites.

species	order	study
Falco sparverius	Falconiformes	Apanius, V. & Kirkpatrick, C.E. 1988. Preliminary report of Haemoproteus tinnunculi infection in a breeding population of American Kestrels. J. Wildl. Dis. 24(1):150-153.
Accipiter nisus	Accipitriformes	Ashford, R.W. et al 1990. Leucocytozoon toddi in British sparrowhawks Accipiter nisus: observations on the dynamics of infection. J. Nat. Hist. 23(5): 1101-1107.
Cardinalis phoeniceus	Passeriformes	Belo, N.O. et al. 2012. Diversity of avian haemosporidians in arid zones of northern Venezuela. Parasitology 139: 1021-1028.
Tiaris bicolor	Passeriformes	Belo, N.O. et al. 2012. Diversity of avian haemosporidians in arid zones of northern Venezuela. Parasitology 139: 1021-1028.
Grus americana	Gruiformes	Bertram, M.R. et al. 2016. Haemosporida prevalence and diversity are similar in endangered wild whooping cranes (Grus americana) and sympatric sandhill cranes (Grus canadensis). Parasitology 144(5): 629-640.
Grus canadensis	Gruiformes	Bertram, M.R. et al. 2016. Haemosporida prevalence and diversity are similar in endangered wild whooping cranes (Grus americana) and sympatric sandhill cranes (Grus canadensis). Parasitology 144(5): 629-640.
Zonotrichia leucophrys	Passeriformes	Bonier, F. et al. 2006. Sex-specific consequences of life in the city. Behav. Ecol. 18(1): 121-129.
Anthus campestris	Passeriformes	Calero-Riestra, M. & García, J.T. 2016. Sex-dependent differences in avian malaria prevalence and consequences of infections on nestling growth and adult condition in the Tawny pipit, Anthus campestris. Malaria Journal 15: 178.
Pica nuttalli	Passeriformes	Clark, G. 1964. Frequency of Infection and Seasonal Variation of Leucocytozoon berestnefi in the Yellow-billed Magpie, Pica nuttalli. The Journal of Protozoology 11(4): 481-484.
Larus scopulinus	Charadriiformes	Cloutier, A. et al. 2011. Plasmodium infections of red-billed gulls (Larus scopulinus) show associations with host condition but not reproductive performance. J. R. Soc. N. Z. 41(4): 261 277.

Meleagris gallopavo	Galliformes	Cook, R.S. et al. 1966. Haemoproteus in Wild Turkeys from the Coastal Bend of South Texas. The Journal of Protozoology 13(4): 588-590.
Ficedula hypoleuca 2	Passeriformes	Dale, S. et al. 1996. Effects of blood parasites on sexual and natural selection in the pied flycatcher. J. Zool. 238(2): 373-393.
Progne subis	Passeriformes	Davidar, P. & Morton, E.S. 1993. Living with parasites: prevalence of a blood parasite and its effect on survivorship in the purple martin. The Auk 110(1):109-116.
Junco hyemalis	Passeriformes	Deviche, P. et al. 2001. Seasonal and Age-Related Changes in BloodParasite Prevalence in Dark-Eyed Juncos (Junco hyemalis, Aves, Passeriformes). J. Exp. Zool. 289(7): 456-466.
Loxia leucoptera	Passeriformes	Deviche, P. et al. 2009. Blood parasitaemia in a high latitude flexible breeder, the white-winged crossbill, Loxia leucoptera: contribution of seasonal relapse versus new inoculations. Parasitology 137(2): 261-273.
Cygnus olor	Anseriformes	Dolka, B. et al. 2014. Hematological parameters in relation to age, sex and biochemical values for mute swans (Cygnus olor). Veterinary Research Communications 38: 93–100.
Ficedula hypoleuca	Passeriformes	Dubiec, A. et al. 2017. Haemoparasites of the pied flycatcher, inter-population variation in the prevalence and community composition. Parasitology 145(7): 912-919.
Parus major	Passeriformes	Dunn, J. et al. 2011. Personality and parasites: sex-dependent associations between avian malaria infection and multiple behavioural traits. Behav. Ecol. Sociobiol. 65:1459-1471.
Quelea quelea	Passeriformes	Durrant, K.L. et al. 2007 Variation in haematozoan parasitism at local and landscapelevels in the red-billed quelea Quelea quelea. J. Avian Biol. 38(6): 662-671.
Baeolophus bicolor	Passeriformes	Fast, K.M. et al. 2016. Haemosporidian prevalence and parasitemia in the tufted titmouse (Baeolophus bicolor). J. Parasitol. 102(6): 636-642.
Acrocephalus schoenobaenus	Passeriformes	Fernandez et al 2010 High prevalence of haemosporidians in Reed Warbler Acrocephalus scirpaceus and Sedge Warbler Acrocephalus schoenobaenus in Spain
Acrocephalus scirpaceus	Passeriformes	Fernandez, M. et al. 2010. High prevalence of haemosporidians in Reed Warbler Acrocephalus scirpaceus and Sedge Warbler Acrocephalus schoenobaenus in Spain. J. Ornothol. 151: 27.
Dendragapus obscurus	Galliformes	Forbes, M. R. et al. 1994. Blood Parasites of Blue Grouse: Variation in Prevalence and Patterns of Interspecific Association. Oecologia 97(4): 520-525.
Dendragapus obscurus 2	Galliformes	Forbes, M. R. et al. 1994. Blood Parasites of Blue Grouse: Variation in Prevalence and Patterns of Interspecific Association. Oecologia 97(4): 520-525.
Passerculus sandwichensis	Passeriformes	Freeman-Gallant, C.R. et al. 2001. Sexual Selection and the Geography of Plasmodium Infection in Savannah Sparrows (Passerculus sandwichensis). Oecologia 127: 517-521.
Cyanocitta cristata	Passeriformes	Garvin, M. & Schoech, S. 2006. Hormone Levels and Infection of Haemoproteus danilewskyi in Free-Ranging Blue Jays (Cyanocitta cristata) J. Parasitol., 92(3): 659-662.
Anas discors	Anseriformes	Garvon, J.M. et al. 2016. Blood Parasites of Blue-winged Teal (Anas discors) from Two Migratory Corridors, in the Southern USA. 52(3): 725-729.
Zenaida macroura	Columbiformes	Godfrey R.D. et al. 1990. Effects of host and spatial factors on a haemoproteid community in mourning doves from western. J. Wildl. Dis. 26(4): 435-441.
Prunella collaris	Passeriformes	Haas, M. & Kisková, J. 2010. Absence of blood parasites in the Alpine Accentor Prunella collaris. Oecologia Montana 19: 30-34.
Arenaria interpres	Charadriiformes	Hargreaves, A.L. et al. 2010. Concentrations of 17 elements, including mercury, and their relationship to fitness measures in arctic shorebirds and their eggs. Science of the Total Environment 408(16): 3153-3161.

Carpodacus mexicanus	Passeriformes	Hartup, B.K. et al. 2008. Blood Parasites of House Finches (Carpodacus mexicanus) from Georgia and New York. J. Wildl. Dis. 44(2): 469-474.
Acrocephalus arundinaceus	Passeriformes	Hasselquist, D. et al. 2007. Temporal patterns of occurrence and transmission of the blood parasite Haemoproteus payevskyi in the great reed warbler Acrocephalus arundinaceus. J. Ornithol. 148: 401-409.
Turdus merula	Passeriformes	Hatchwell, B.J. et al. 2000. The prevalence and ecology of the haematozoan parasites of European blackbirds, Turdus merula. Canadian Journal of Zoology 78(4): 684-687.
Lagopus lagopus	Galliformes	Holmastad, P.R. & Skorping A. 1998. Covariation of parasite intensities in willow ptarmigan, Lagopus lagopus L. Canadian Journal of Zoology 76(8): 1581-1588.
Accipiter soloensis	Accipitriformes	Hsu, Y.C. et al. 2015. Prevalence of Blood Parasites in Three Migratory Raptor Species from Taiwan J. Raptor Res. 49(2): 227-230.
Butastur indicus	Accipitriformes	Hsu, Y.C. et al. 2015. Prevalence of Blood Parasites in Three Migratory Raptor Species from Taiwan J. Raptor Res. 49(2): 227-230.
Falco tinnunculus	Falconiformes	Hsu, Y.C. et al. 2015. Prevalence of Blood Parasites in Three Migratory Raptor Species from Taiwan J. Raptor Res. 49(2): 227-230.
Dendragapus canadensis	Galliformes	Jones, T.L. & Robinson, W.L. 1969. Blood parasites of Michigan spruce grouse, Canachites canadensis. J. Parasitol. 55(3): 492.
Falco tinnunculus 2	Falconiformes	Korpimaki, E. et al. 1995. Blood parasites, sexual selection and reproductive success of European kestrels. Ecoscience 2(4): 335-343/
Aegolius funereus	Strigiformes	Korpimdki, E. et al. 1993. Blood parasites and reproductive success of Tengmalm's owls: detrimental effects on females but not on males? Functional Ecology 7: 420-426.
Parus cinctus	Passeriformes	Krams, I. et al. 2010. Effects of forest management on haematological parameters, blood parasites, and reproductive success of the Siberian tit (Poecile cinctus) in northern Finland. Annales Zoologici Fennici 47(5): 335-346.
Ficedula albicollis	Passeriformes	Kulma, K. et al. 2013. Malaria infections reinforce competitive asymmetrybetween two Ficedula flycatchers in a recent contact zone. Mol. Ecol. 22(17): 4591-4601.
Anas discors 2	Anseriformes	Loven, J.S. et al. 1980. Blood parasitemia in a south Texas wintering waterfowl population. J. Wildl. Dis. 16: 25-28.
Carduelis tristis	Passeriformes	Lumpkin, D.C. et al. 2014. Blood parasite infection differentially relates to carotenoid-based plumage and bill color in the American goldfinch. Ecol. Evol. 4(16): 3210-3217.
Fulmarus glacialis	Procelariiformes	Mallory, M.L. et al. 2007. Breeding status, contaminant burden and helminth parasites of Northern Fulmars Fulmarus glacialis from the Canadian high Arctic. Ibis 149(2): 338-344.
Charadrius alexandrinus	Charadriiformes	Martínez-de la Puente, J. et al. 2017. Extremely low Plasmodium prevalence in wild plovers and coursers from Cape Verde and Madagascar. Malaria Journal 16: 243.
Charadrius marginatus	Charadriiformes	Martínez-de la Puente, J. et al. 2017. Extremely low Plasmodium prevalence in wild plovers and coursers from Cape Verde and Madagascar. Malaria Journal 16: 243.
Charadrius pecuarius	Charadriiformes	Martínez-de la Puente, J. et al. 2017. Extremely low Plasmodium prevalence in wild plovers and coursers from Cape Verde and Madagascar. Malaria Journal 16: 243.
Charadrius thoracicus	Charadriiformes	Martínez-de la Puente, J. et al. 2017. Extremely low Plasmodium prevalence in wild plovers and coursers from Cape Verde and Madagascar. Malaria Journal 16: 243.

Cursorius cursor	Charadriiformes	Martínez-de la Puente, J. et al. 2017. Extremely low Plasmodium prevalence in wild plovers and coursers from Cape Verde and Madagascar. Malaria Journal 16: 243.
Larus michahellis	Charadriiformes	Martínez-Abraín, A. et al. 2002. Prevalence of blood parasites in two western-Mediterranean local populations of the Yellow-legged Gull Larus cachinnans michahellis. Ornis Fenn. 79: 34-40.
Philomachus pugnax	Charadriiformes	Mendes, L. et al 2013. Hidden haemosporidian infections in Ruffs (Philomachus pugnax) staging in Northwest Europe en route from Africa to Arctic Europe. Parasitol. Res. 112(5): 2037-2043.
Carduelis chloris	Passeriformes	Merila, J. et al. 1995. Geographic and individual variation in haematozoan infections in the greenfinch, Carduelis chloris. Canadian Journal of Zoology 73(10):1798-1804.
Parus caeruleus	Passeriformes	Merila, J. & Andersson, M. 1999. Reproductive effort and success are related to haematozoan infections in blue tits. Ecoscience 6(3): 421-428.
Anas penelope	Anseriformes	Mohammad, M.K. 2015. The Parasitic Fauna of the Wigeon Anas penelope L. 1758 Collected in central Iraq. Int. J. Adv. Res. Biol. Sci. 3(2): 243-246.
Acrocephalus arundinaceus 2	Passeriformes	Nagata, H. & Sodhi, N. 2003. Low prevalence of blood parasites in five Sylviidae species in Japan. Ornithol. Sci. 2: 73–74
Acrocephalus bistrigiceps	Passeriformes	Nagata, H. & Sodhi, N. 2003. Low prevalence of blood parasites in five Sylviidae species in Japan. Ornithol. Sci. 2: 73–74
Cisticola juncidis	Passeriformes	Nagata, H. & Sodhi, N. 2003. Low prevalence of blood parasites in five Sylviidae species in Japan. Ornithol. Sci. 2: 73–74
Locustella pryeri	Passeriformes	Nagata, H. & Sodhi, N. 2003. Low prevalence of blood parasites in five Sylviidae species in Japan. Ornithol. Sci. 2: 73–74
Acrocephalus paludicola	Passeriformes	Neto, J.M. et al. 2015. Prevalence and diversity of Plasmodium and Haemoproteus parasites in the globally-threatened Aquatic Warbler Acrocephalus paludicola. Parasitology 142(9): 1183-1189.
Aix sponsa	Anseriformes	O'Dell, J.P. & Robbins, L.W. 1994. Hematozoa of wood ducks (Aix sponsa) in Missouri. J. Wildl. Dis 30(1): 36-39.
Aix sponsa 2	Anseriformes	O'Dell, J.P. & Robbins, L.W. 1994. Hematozoa of wood ducks (Aix sponsa) in Missouri. J. Wildl. Dis 30(1): 36-39.
Fringilla coelebs	Passeriformes	Pawelczyk, A. et al. 2003. Parasites of chaffinch (Fringilla coelebs) population. Part ii. Blood parasites. Wiadomości parazytologiczne 49(1):31-38.
Delichon urbicum	Passeriformes	Piersma, T. & van der Velde, M. 2012. Dutch House Martins Delichon urbicum gain blood parasite infections over their lifetime, but do not seem to suffer. J Ornithol. 153: 907-912.
Picoides borealis	Piciformes	Pung, O.J. et al. 2000. Survey and host fitness effects of red-cockaded woodpecker blood parasites and nest cavity arthropods. J Parasitol 86(3):506-510.
Cinclus cinclus	Passeriformes	Rojo, M.A. et al. 2013. Prevalence of haematozoan parasites in the White-throated Dipper Cinclus cinclus in southern Europe. Bird Study 60(2): 247-256.
Dendroica coronata	Passeriformes	Rooney, L. 2015. Natural variation in malarial infection and immune investment in a migratory songbird, and the effects of infection on flight performance. Thesis (M.Sc.), University of Western Ontario. Canada.
Larus audouinii	Charadriiformes	Ruiz, X. et al. 1995. Incidence of a Haemoproteus lari parasitemia in a threatened Gull: Larus audouinii. Ornis Fennica 72: 159-164.
Parus montanus	Passeriformes	Rytkonen, S. et al. 1996. Absence of blood parasites in Willow Tits Parus montanus in northern Finland. J. Avian Biol. 27(2): 173-174.

Sylvia atricapilla	Passeriformes	Santiago-Alarcón, D. et al. 2011. Prevalence, diversity, and interaction patterns of avian haemosporidians in a four-year study of blackcaps in a migratory divide. Parasitology 138(7): 824-835.
Melanerpes carolinus	Piciformes	Schrader, M.S. et al. 2003. Seasonal prevalence of a haematozoan parasite of red-bellied woodpeckers (Melanerpes carolinus) and its association with host condition and overwinter survival. The Auk 120(1):130-137.
Ploceus capensis	Passeriformes	Schultz, A. et al. 2010. Infection prevalence and absence of positive correlation between avian haemosporidian parasites, mass and body condition in the Cape Weaver Ploceus capensis. Ostrich 81(1): 69-76.
Ploceus capensis 2	Passeriformes	Schultz, A. et al. 2010. Infection prevalence and absence of positive correlation between avian haemosporidian parasites, mass and body condition in the Cape Weaver Ploceus capensis. Ostrich 81(1): 69-76.
Carduelis flammea	Passeriformes	Seutin, G. 1994. Plumage redness in redpoll finches does not reflect hemoparasitic infection. Oikos 70(2): 280-286.
Forpus passerinus	Psittaciformes	Sheridan, J.A. et al. 2004. Weak association between measures of health and reproductive success in green-rumped parrotlets (Forpus passerinus) in Venezuela. The Auk 121(3): 717-725.
Icteria virens	Passeriformes	Soares, L. et al. 2016. Co-infections of haemosporidian and trypanosome parasites in a North American songbird. Parasitology 143(14): 1930-1938.
Columba livia	Columbiformes	Sol, D. et al. 2000. Geographical variation in blood parasites in feral pigeons: the role of vectors. Ecography 23: 307-314.
Centrocercus urophasianus	Galliformes	Stabler, R.M. et al. 1977. Hematozoa in sage grouse from Colorado. J. Wildl. Dis. 13(4): 414-417.
Centrocercus urophasianus 2	Galliformes	Stabler, R.M. et al. 1977. Hematozoa in sage grouse from Colorado. J. Wildl. Dis. 13(4): 414-417.
Emberiza citrinella	Passeriformes	Sundberg, J. 1995. Parasites, plumage coloration and reproductive success in the yellowhammer, Emberiza ctrinella. Oikos 74(2): 331-339.
Accipiter striatus	Accipitriformes	Taft, S.J. et al. 1996. Hematozoa in Autumnal Migrant Raptors from the Hawk Ridge Nature Reserve, Duluth, Minnesota. Helminthol. Soc. Wash. 63(1): 141-143.
Accipiter cooperii	Accipitriformes	Taft, S.J. et al. 1994. Avian hematozoa of adult and nestling Cooper's Hawks (Accipiter cooperii) in Wisconsin. J. Helminthol. Soc. Wash. 61(1): 146-148.
Accipiter cooperii 2	Accipitriformes	Taft, S.J. et al. 1996. Hematozoa in Autumnal Migrant Raptors from the Hawk Ridge Nature Reserve, Duluth, Minnesota. Helminthol. Soc. Wash. 63(1): 141-143.
Accipiter gentilis	Accipitriformes	Taft, S.J. et al. 1996. Hematozoa in Autumnal Migrant Raptors from the Hawk Ridge Nature Reserve, Duluth, Minnesota. Helminthol. Soc. Wash. 63(1): 141-143.
Falco naumanni	Falconiformes	Tella, J.L. et al. 1996. Absence of blood-parasitization effects on Lesser Kestrel fitness. The Auk 113(1): 253-256
Troglodytes troglodytes	Passeriformes	Topp, S.M. et al. 2007. Apparent absence of blood parasites in Winter Wrens in British Columbia. J. Field Ornithol. 78(3):308 313
Bucanetes githagineus	Passeriformes	Valera, F. et al. 2003. Low prevalence of haematozoa in Trumpeter finches Bucanetes githagineus from south-eastern Spain: additional support for a restricted distribution of blood parasites in arid lands. Journal of Arid Environments 55: 209-213.
Coccothraustes coccothraustes	Passeriformes	Valkiūnas, G. et al. 2003. High prevalence of blood parasites in hawfinch Coccothraustes coccothraustes. Journal of Natural History 37:2647-2652.
Motacilla flava	Passeriformes	Valkiūnas, G. & Iezhova, T.A. 2001. A Comparison of the Blood Parasites in Three Subspecies of the Yellow Wagtail Motacilla flava. Journal of Parasitology 87(4): 930-934.

Agelaius phoeniceus	Passeriformes	Weatherhead, P.J. & Bennett, G.F. 1991. Ecology of Red-winged Blackbird parasitism by haematozoa. Canadian Journal of Zoology 69(9): 2352-2359.
Molothrus ater	Passeriformes	Weatherhead, P.J. & Bennett, G.F. 1992. Ecology of parasitism of Brown-headed Cowbirds by haematozoa. Canadian Journal of Zoology 70(1): 1-7.
Dendroica coronata 2	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Dendroica magnolia	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Dendroica striata	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Mniotilta varia	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Oporornis philadelphia	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Seiurus aurocapilla	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Seiurus noveboracensis	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Setophaga ruticilla	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Vermivora peregrina	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Wilsonia pusilla	Passeriformes	Weatherhead, P.J. et al. 1991. Sexual selection and parasites in wood-warblers. The Auk 108(1): 147-152.
Parus atricapillus	Passeriformes	Wilkinson, L.C. et al. 2016. Avian malaria in a boreal resident species: long-term temporal variability, and increased prevalence in birds with avian keratin disorder. Int. J. Parasitol. 46(6): 281-290.
Fregata minor	Suliformes	Work, T.M. & Rameyer, R.A. 1996. Haemoproteus iwa n. sp. in Great Frigatebirds (Fregata minor [Gmelin]) from Hawaii: Parasite Morphology and Prevalence. J. Parasitol. 82(3): 489-491.
Calidris melanotos	Charadriiformes	Yohannes, E. et al. 2009. Prevalence of malaria and related haemosporidian parasites in two shorebird species with different winter habitat distribution. J. Ornithol. 150: 287-291.
Calidris pusilla	Charadriiformes	Yohannes, E. et al. 2009. Prevalence of malaria and related haemosporidian parasites in two shorebird species with different winter habitat distribution. J. Ornithol. 150: 287-291.
Larus argentatus	Charadriiformes	Zagalska-Neubauer, M. et al. 2016. High prevalence of Leucocytozoon parasites in fresh water breeding gulls. J. Ornithol. 157: 525-532.

Table S2. List of 39 studies consulted to extract data on gastrointestinal parasites.

species	order	study
Galerida_cristata	Passeriformes	Al-Ankari, A.-R.S. et al. 2003. First Report of Variolepis farciminosa (Cestoda: Hymenolepididae) and Diplotriaena tridens (Nematoda: Diplotriaenoidea) Infecting Crested Larks, Galerida cristata, from Hofuf, Al-Ahsa Oasis, Saudi Arabia. Comp. Parasitol. 70(1): 97-98.
Larus_michahellis	Charadriiformes	Álvarez, M.F. et al. 2006. Influence of host age and sex on the helminth fauna of the yellow-legged gull Larus Michahellis in Galicia Northwestern Spain. J Paratisol. 92(3): 454-458.
Pica_pica	Passeriformes	Amin, O.M. et al. 2010. Redescription of Sphaerirostris picae (Acanthocephala: Centrorhynchidae) from magpie, Pica pica, in Northern Iran, with special reference to unusual receptacle structures and notes on histopathology. 96(3): 561-568.

Anser_albifrons	Anseriformes	Amundson, C.L. et al. 2016. Helminth community structure in two species of arctic-breeding waterfowl. Int. J. Parasitol. Parasites Wildl. 5(3): 263-272.
Branta_bernicla	Anseriformes	Amundson, C.L. et al. 2016. Helminth community structure in two species of arctic-breeding waterfowl. Int. J. Parasitol. Parasites Wildl. 5(3): 263-272.
Haematopus_ostralegus	Charadriiformes	Borgsteede, F.H.M. et al. 1988. Helminth parasites of the digestive tract of the oystercatcher, Haematopus ostralegus, in the Wadden Sea, The Netherlands. Neth. J. Sea Res. 22(2): 171-174.
Uria_aalge	Charadriiformes	Brosens, L. et al. 1996. Observations on the helminths of harbour porpoises (Phocoena phocoena) and common guillemots (Uria aalge) from the Belgian and German coasts. Vet. Record 139(11): 254-257.
Carduelis_chloris	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Emberiza_citrinella	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691..
Fringilla_coelebs	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Parus_caeruleus	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Parus_major	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Passer_domesticus	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Phylloscopus_trochilus	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Prunella_modularis	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Regulus_regulus	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Sturnus_vulgaris	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Sylvia_atricapilla	Passeriformes	Brown, M.A. et al. 2010. Coccidian parasites of British wild birds. J. Nat. Hist. 44(43-44): 2669-2691.
Anas_acuta	Anseriformes	Crichton, V.F.J. & Welch, H.E. 1972. Helminths from the digestive tracts of mallards and pintails in the Delta Marsh, Manitoba. Canadian Journal of Zoology 50(5): 633-637.
Anas_platyrhynchos	Anseriformes	Crichton, V.F.J. & Welch, H.E. 1972. Helminths from the digestive tracts of mallards and pintails in the Delta Marsh, Manitoba. Canadian Journal of Zoology 50(5): 633-637.
Callipepla_squamata	Galliformes	Dunham, N.R. & Kendall, R.J. 2016. Eyeworm infections of Oxyspirura petrowi, Skrjabin, 1929 (Spirurida: Thelaziidae), in species of quail from Texas, New Mexico and Arizona, USA. J. Helminthol. 91(4): 491-496.
Colinus_virginianus	Galliformes	Dunham, N.R. et al. 2014. Evidence of an Oxyspirura petrowi epizootic in northern bobwhites (Colinus virginianus), Texas, USA. J. Wildl. Dis. 50(3): 552-558.
Quiscalus_quiscula	Passeriformes	Fayer, R. & Kocan, R.M. 1971. Prevalence of Sarcocystis in Grackles in Maryland. Journal of Protozoology 18(3): 547-548.
Chen_caerulescens	Anseriformes	Gajadhar, A. et al. 1983. Prevalence of renal coccidia in wild waterfowl in Saskatchewan. Canadian Journal of Zoology 61(11): 2631-2633.
Dendrocygna_autumnalis	Anseriformes	George, R.R. & Bolen, E.G. 1975. Endoparasites of black-bellied whistling ducks in Southern Texas. J. Wildl. Dis. 11(1): 17-22.
Phasianus_colchicus	Galliformes	Gethings, O.J. et al. 2016. Body condition is negatively associated with infection with Syngamus trachea in the ring-necked pheasant (Phasianus colchicus). Vet. Parasitol. 228: 1-5.

Anas_crecca	Anseriformes	Green, A.J. et al. 2011. Determinants of the prevalence of the cloacal cestode Cloacotaenia megalops in teal wintering in the French Camargue. Eur. J. Wildl. Res. 57: 275–281.
Larus_argentatus	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Larus_argentatus_2	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Larus_fuscus	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Larus_fuscus_2	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Larus_marinus	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Larus_marinus_2	Charadriiformes	Gysels, H. & Rabaey, M. 1964. The incidence of some species of trematoda in three species of Larus gulls in Wales. Ibis 106(4): 532-540.
Tympanuchus_cupido	Galliformes	Harper, G.R. et al. 1967. Helminths of greater prairie chickens in Kansas. The Journal of Wildlife Management 31(2): 265-269.
Lagopus_lagopus	Galliformes	Holmastad, P.R. & Skorping A. 1998. Covariation of parasite intensities in willow ptarmigan, Lagopus lagopus L. Canadian Journal of Zoology 76(8): 1581-1588.
Tetrao_tetrix	Galliformes	Isomursu, M. et al. 2006. Sex and age influence intestinal parasite burden in three boreal grouse species. Journal of Avian Biology, 37: 516-522
Tetrao_urogallus	Galliformes	Isomursu, M. et al. 2006. Sex and age influence intestinal parasite burden in three boreal grouse species. Journal of Avian Biology, 37: 516-522
Phalacrocorax_carbo	Suliformes	Kanarek, G. & Zalesny, G. 2013. Extrinsic- and intrinsic-dependent variation in component communities and patterns of aggregations in helminth parasites of great cormorant (Phalacrocorax carbo) from N.E. Poland. Parasitol. Res. 113: 837-850.
Larus_delawarensis	Charadriiformes	Levy, M.S. 1997. Helminth communities of ring-billed gull (Larus delawerensis) collected along the St. Lawrence River and Estuary. Thesis (M.Sc.), Concordia University. Canada.
Tyrannus_tyrannus	Passeriformes	Mackenzie, D.I. et al. 1979. Comparison of the helminth fauna of eastern and western kingbirds at Delta Marsh, Manitoba. Canadian Journal of Zoology 57(5): 1143-1149.
Tyrannus_verticalis	Passeriformes	Mackenzie, D.I. et al. 1979. Comparison of the helminth fauna of eastern and western kingbirds at Delta Marsh, Manitoba. Canadian Journal of Zoology 57(5): 1143-1149.
Fulmarus_glacialis	Procellariiformes	Mallory, M.L. et al. 2007. Breeding status, contaminant burden and helminth parasites of Northern Fulmars Fulmarus glacialis from the Canadian high Arctic. Ibis 149(2): 338-344.
Fulmarus_glacialis_2	Procellariiformes	Mallory, M.L. et al. 2007. Breeding status, contaminant burden and helminth parasites of Northern Fulmars Fulmarus glacialis from the Canadian high Arctic. Ibis 149(2): 338-344.
Strix_aluco	Strigiformes	McInes, F.J. et al. 1994. The distribution of Centrorhynchus aluconis (Acanthocephala) and Porrocaecum spirale (Nematoda) in tawny owls (Strix aluco) from Great Britain. J. Raptor Res. 28(1): 34-38.
Anas_platyrhynchos_2	Anseriformes	Mercado-Reyes, M. et al. 2010. Presence of helminths in the Mexican duck Anas platyrhynchos diazi of the Zacatecano Plateau, Mexico. Agrociencia 44(8): 931-939.

Corvus_frugilegus	Passeriformes	Mettrick, D.F. 1960 Helminth Parasites of Hertfordshire Birds—IV Survey Results. Journal of Helminthology 43(3-4): 267-276.
Corvus_monedula	Passeriformes	Mettrick, D.F. 1960 Helminth Parasites of Hertfordshire Birds—IV Survey Results. Journal of Helminthology 43(3-4): 267-276.
Alectoris_rufa	Galliformes	Millán, J. et al. 2004. Ecology of nematode parasitism in red-legged partridges (Alectoris rufa) in Spain. Helminthologia 41(1): 33-37.
Anas_penelope	Anseriformes	Mohammad, M.K. 2015. The Parasitic Fauna of the Wigeon Anas penelope L. 1758 Collected in central Iraq. Int. J. Adv. Res. Biol. Sci. 3(2): 243-246.
Sturnus_vulgaris_2	Passeriformes	Moore, J. & Bell, D.H. 1983. Pathology (?) of Plagiorhynchus cylindraceus in the starling, Sturnus vulgaris. J. Parasitol. 69(2): 387-390.
Callipepla_californica	Galliformes	Moore, J. et al. 1988. Dispharynx nasuta (Nematoda) in California Quail (Callipepla californica) in Western Oregon. J. Wildl. Dis. 24(3): 564-567.
Branta_canadensis	Anseriformes	Nowicki, A. et al. 1995. Gizzard nematodes of Canada geese wintering in Southern Illinois. J. Wildl. Dis. 31(3): 307-313.
Sialia_sialis	Passeriformes	Carleton, R.E. 2007. Disease ecology of a population of eastern bluebirds (Sialia sialis) in Georgia: a starting point. Thesis (PhD), University of Georgia. USA.
Sula_nebouxii	Suliformes	Rubio-Godoy, M. et al. 2011. Helminth Parasites of the Blue-Footed Booby on Isla Isabel, México. J. Parasitol. 97(4): 636-641.
Aythya_fuligula	Anseriformes	Rzad, I. et al. 2012. Digenean communities in the tufted duck [Aythya fuligula (L., 1758)] and greater scaup [A. marila (L., 1761)] wintering in the north-west of Poland. J. Helmonthol. 87(2): 230-239.
Aythya_marila	Anseriformes	Rzad, I. et al. 2012. Digenean communities in the tufted duck [Aythya fuligula (L., 1758)] and greater scaup [A. marila (L., 1761)] wintering in the north-west of Poland. J. Helmonthol. 87(2): 230-239.
Scolopax_rusticola	Charadriiformes	Sanchez-Garcia, C. et al. 2017. Is cestode infection intensity associated with decreased body condition in the Eurasian woodcock Scolopax rusticola? J. Helminthol. 92(1): 42-48.
Vanellus_chilensis	Charadriiformes	Silveira, T.S. & Calegaro-Marques, C. 2016. Helminth parasite diversity discloses age and sex differences in the foraging behaviour of southern lapwings Vanellus chilensis. Austral Ecology 41(5): 549-558.
Ciconia_ciconia	Ciconiiformes	Sitko, J. & Heneberg, P. 2015. Composition, structure and pattern of helminth assemblages associated with central European storks (Ciconiidae). Parasitology International 64(2): 130-134.
Anas_crecca_2	Anseriformes	Turner, B.C. & W. Threlfall. 1975. The Metazoan parasites of Green-winged Teal (Anas crecca L.) and Blue-winged Teal (Anas discors L.) from eastern Canada. Proc. Helminthol. Soc. Wash .42: 157-169.
Columba_livia	Columbiformes	Vaz, FF. et al. 2017. Gastrointestinal helminths of two populations of wild pigeons Columba livia in Brazil. Rev. Bras. Parasitol. Vet. 26(4): 446-450.

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Chapter 4 | Sex-specific immune function in shorebirds

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This paper reports on original research I conducted during the period of my Higher Degree by Research candidature

4.1 Abstract

Work in the field imposes many limitations that could potentially constrain effective sampling of tissue that may require strict conditions of collection or transportation, often used in determination of many physiological parameters. The immune system is critical for infection defence and several theories postulate trade-offs with life-history traits, which could help answer yet unknown questions about different mortality rates of males and females in birds. However, relatively little is known about immunity in male and female shorebirds and how it might differ at different age groups. Here we quantify immune function in two species of shorebirds, the Kentish plover (*Charadrius alexandrinus*), in which males survive better than females so that one would expect males to have superior immune function than females, and the monogamous Black-winged stilt (*Himantopus himantopus*), with no sex differences expected. Using samples from wild birds, we determined sex-specific agglutination and lysis titres in 30 adult Kentish plovers and 21 Black-winged stilts chicks. Contrary to our expectations, we found no sexual differences in immune function between males and females in Kentish plover. Black-winged stilts also lacked sex differences in immunity, although these were in line with the expectation. In addition, body condition of neither Kentish plover nor Black-winged stilts were related to the strength of their immune function. Taken together, our results provide partial support to the hypothesised link between sex different survival and immune function. Further samples –possibly from additional shorebird species of both chicks and adults– are needed to investigate the hypothesised relationship between survival and immune function.

Keywords: immunocompetence, immunity, sex bias, sexual selection, waders, mating strategy

4.2 Introduction

The study of wildlife in their environment has enabled the understanding of crucial patters of the natural world. However, work in the field is often constrained by a number of factors that go beyond our control such as weather, local facilities (e.g. electricity) or geographical features. One meaningful variable to evaluate in wild populations is the immune system because is the main defence mechanism against exogenous challenges such as pathogens (Abbas et al., 2015). There are several methods for assessing the strength of the immune system in birds in the field, most of them routinely conducted in passerines (for examples, see Horrocks et al., 2015; and Nwaogu et al., 2019). They are generally based on blood sampling and critically depend on prompt access to refrigeration and/or below-zero-degrees (0°C) storage. However, despite following strict protocols, the consistency of some of these immune assays seems to vary between species (AH pers. obs.). The haemagglutination and haemolysis assays are two immune assays that evaluate baseline immune function by quantifying natural antibodies and complement activity from plasma, respectively. Since their introduction as a reliable measure of innate immune function (Matson et al., 2005), these assays have been used extensively in many bird species but rarely in shorebirds. Because Matson et al. (2005) recognised significant variation among species in these assays, the lack of studies available using this method could be a source of concern in terms of reliability for researches that specialise in this bird group.

In the present work we test the effectivity in the field of two well-stablished immune assays, the haemagglutination and haemolysis assay, to evaluate aspects of the innate immune defence in two shorebird species with no prior records with such tests. We specifically aimed to conduct these analyses in Kentish plovers (*Charadrius alexandrinus*) and Black-winged stilts chicks (*Himantopus himantopus*) breeding sympatrically in Southern Spain, taking into consideration sex differences and age classes. We generated immune function predictions based on the birds' mating system and its association with intra-sexual agonistic behaviour. In polygamous systems the sexes are believed to be under stronger sexual selection forces that in turn will select for traits that will optimise mate acquisition, often including physical or behavioural traits such as ornamentation and vocalisation (Grether, 1996). An example of this is the agonistic behaviour, commonly shown by males against other males while holding territory or to get access to females. However, competing for these resources comes at high costs as frequently result in physical injury or even death (Liu et al., 2017).

Under these assumptions, in the polyandrous Kentish plover, we expect males to present a stronger innate immune defence as an adaptive result of intense male-male competition (fights). Whereas in the monogamous Black-winged stilt, we would expect no differences in immune defence between the sexes. In both species we also accounted for possible confounding effects related to body condition and developmental stage (Stambaugh et al., 2011; De Rosa et al., 2015). Furthermore, demographic variables in Kentish plover support the direction of the sex bias since they present a balanced hatching sex ratio, male-biased adult sex ratio and female-biased adult mortality (Székely et al., 2004; Eberhart-Phillips et al., 2018; Que et al., 2019). Black-winged stilts are seasonally monogamous (Cuervo, 2003) and suspected to have balanced adult sex ratio

(based on life-history traits and other members of the genus, Robinson & Oring, 1996; Figuerola, 1999), suggesting that selection modulates immune strength equally between the sexes, possibly even before reaching adulthood (suggested by equal male and female chick mortality, see Eberhart-Phillips et al., 2018).

4.3 Materials and methods

4.3.1 *Bird species and sampling locations*

Kentish plovers are small (35–55 g) ground nesting birds that usually breed near seacoasts, but also open, flat, scarcely vegetated lands near brackish, saline lakes, lagoons, or seasonal water courses across Eurasia. They present small sexual dichromatism during the breeding season, based on males having more striking colouration on feathers around the neck and head compared to females (del Hoyo et al., 2020). Black-winged stilts share the same breeding habitat that Kentish plover, but stilts are more widely distributed, inhabiting areas across the Mediterranean and sub-Saharan Africa to SE Asia and Taiwan. Adult Black-winged stilts may present subtle sexual dichromatism, but in the field the sexes are indissociable (Pierce & Kirwan, 2020).

We captured, ringed, weighed and morphometrically measured breeding birds in two different locations of Southern Spain. In Cádiz Bay Natural Park, Puerto Real (permit number: 2019-/2979/4202/Bc/EA 3619), we studied Kentish plovers in a 35-ha saltpan during May 2019. The second location corresponded to the largest area of rice fields in Spain (36,000 ha) located in a reclaimed marshland behind Doñana National Park (permit number: 2011_02 21/02/2012/77), where we studied Kentish plovers and stilts in June and July 2019 during the peak of the breeding season. In total, we captured 17 female and 13 male adult Kentish plovers, and 11 female and 10 male Black-winged stilt chicks (**Table 4.1**).

Table 4.1 Measures of immune function in adult Kentish plovers and Black-winged stilt chicks.

			Agglutination (titre)		Lysis (titre)	
	Sex	N	Mean	Std. deviation	Mean	Std. deviation
Kentish plover	males	13	4.63	2.27	0.92	1.14
	females	17	3.90	2.44	0.96	1.23
	total	30	4.22	2.36	0.94	1.17
Black-winged stilt	males	10	3.72	2.16	0.27	0.79
	females	11	4.89	1.49	1.34	1.45
	total	21	4.33	1.89	0.83	1.28

4.3.2 *Immune parameters*

From each bird and shortly after capture, we took approx. 60–120 µl of blood from the basilic or jugular vein. The samples were momentarily stored cold to then be centrifuged at 7.000 RPM for 10 min to obtain plasma. Plasma samples were stored frozen until its analysis. We used a haemolysis-haemagglutination assay (rabbit

red blood cells, SB-0009H; ENVIGO) to quantify titres of complement-like lytic enzymes (i.e. lysis) and non-specific natural antibodies (i.e. agglutination) in plasma (Matson et al., 2005; Hegemann et al., 2012). Scans of individual samples were randomised among all plates and scored blindly with respect to sample ID. A plasma standard was run in duplicate in all plates.

Stress of capture has been described to negatively affect immune function (Matson et al., 2006). In Kentish plover, the mean time between capture and sampling was 16.6 min (median = 12 min, min = 4, max = 48), while Black-winged stilts averaged 14.4 min (median = 14 min, min = 7, max = 26). Our data suggests that immune function was unrelated to sampling time in Kentish plovers and Black-winged stilts (Pearson correlation, always *P*-value >0.4; **Fig. S4.1**).

4.3.3 *Molecular sexing*

Male and female Black-winged stilt chicks are sexually monomorphic. Molecular sexing was performed through amplification of the CHD1 gene using the primers P2550F and P2718R (Fridolfsson & Ellegren, 1999), following the PCR protocol proposed by dos Remedios et al. (2010).

4.3.4 *Statistical analysis*

Sexual differences in immunity were tested by running general linear mixed models using the R package *nlme* (Pinheiro et al., 2020). We built separate models for each species. Models for Kentish plovers were fitted with Gaussian error distribution, haemagglutination and haemolysis as response variables, and sex as explanatory variables. Because nutritional state could relate to immune strength (De Rosa et al., 2015), we also added body condition to our model estimated as the scaled mass index (see Peig & Green, 2009). From this model, one female and one male were removed because data on wing length was not available. Models of stilts had a Gaussian error distribution, haemagglutination and haemolysis as response variables, and sex as explanatory variables. Stilt chicks sampled included individuals at different developmental stages, estimated from their body mass to be between 11–29 days of age according to Reed et al. (1999). Therefore, we added the variable body mass as indicator of chick age (Reed et al., 1999) in the models of this species because developmental stage could influence immune capacity (Stambaugh et al., 2011). All models had plate number as random effect variable.

4.4 Results

All plasma samples kept in good condition, allowing successful analysis. The reaction of the plasma with the rabbit blood provided measurable readings.

Contrary to our hypothesis predictions, we found that adult Kentish plovers showed no sex differences in the immune assays, with agglutination titres in males and females Kentish plover of 4.63 and 3.90, respectively, and lysis titres nearly identical between the sexes (**Fig. 4.1**, **Table 4.2**). Although male Kentish plovers had slightly higher haemagglutination titres than females (**Table 4.1**). Black-winged stilts chicks also showed no significant sex differences in agglutination (females = 3.72 and males = 4.89). Again, lysis titres

showed no difference between the sexes (**Table 4.1** and **4.2**), but 8 out of 10 male chicks had no lysis reaction (tires equal zero). It is noteworthy that in most cases the standard deviation was rather high, indicating that the values were spread out over a wide range.

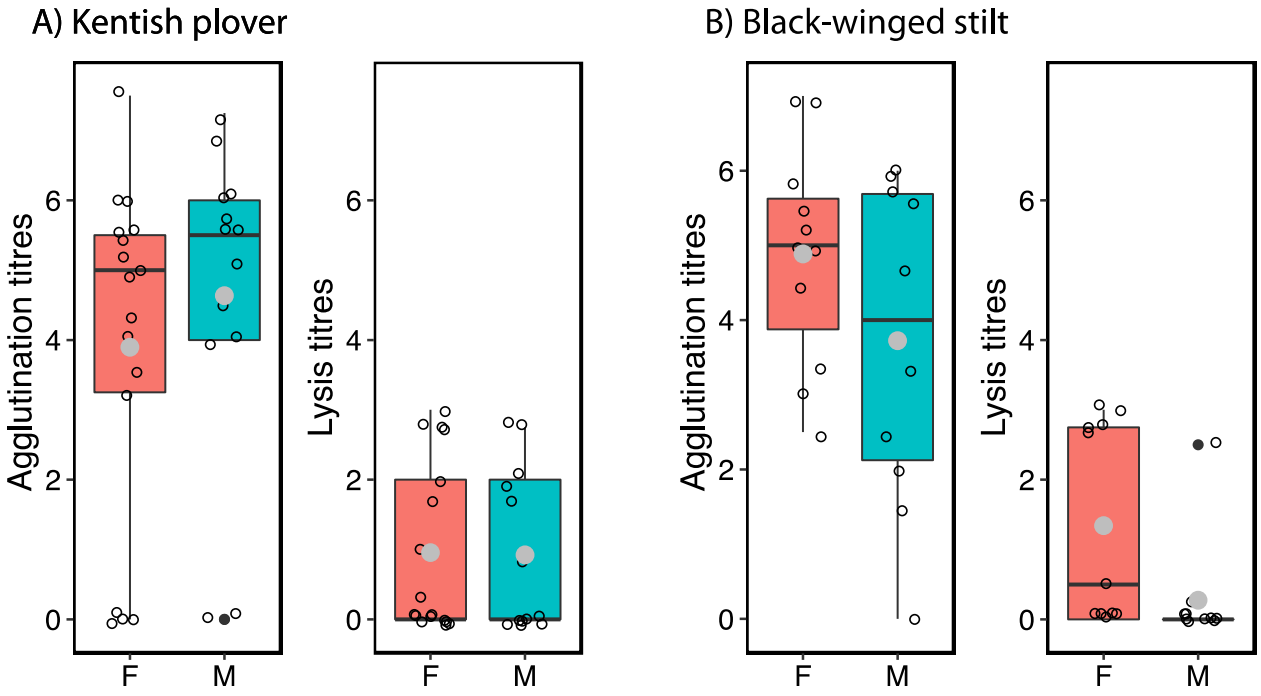

Figure 4.1 Agglutination and lysis results in female and male (a) adult Kentish plovers (n = 30) and (b) Black-winged stilt chicks (n = 21). Medians, upper and lower quartiles are shown. Whiskers indicate minimum and maximum values, solid circles are outliers and open circles the raw data. Grey dots represent arithmetic means.

Body condition in Kentish plover and body mass in stilts had no association with haemagglutination and haemolysis (**Table 4.2**).

Table 4.2 Sex differences in immune function in (a) adult Kentish plovers and (b) Black-winged stilts chicks.

	Estimate	Std. Error	T-stat	*P*-value
a) Kentish plovers (n = 28)				
Haemagglutination assay				
Intercept	4.349	11.829	0.368	0.718
Sex (male)[a]	0.635	1.056	0.601	0.556
Body condition	-0.012	0.272	-0.045	0.964
Random	Variance	Std. Dev		
Plate	0.574	0.758		
Residual	5.619	2.370		
Haemolysis assay				
Intercept	6.147	5.760	1.067	0.302
Sex (male)[a]	-0.136	0.529	-0.257	0.801
Body condition	-0.121	0.132	-0.911	0.376

Random	Variance	Std. Dev		
Plate	<0.001	<0.001		
Residual	1.485	1.219		
b) Black-winged stilt chicks (n = 21)				
Haemagglutination assay				
Intercept	4.272	1.648	2.592	0.025
Sex (male)[a]	-1.185	0.825	-1.435	0.179
Body mass	0.006	0.016	0.397	0.699
Random	Variance	Std. Dev		
Plate	<0.001	<0.001		
Residual	3.550	1.884		
Haemolysis assay				
Intercept	1.936	0.994	1.947	0.078
Sex (male)[a]	-0.911	0.502	-1.814	0.097
Body mass	-0.007	0.009	-0.784	0.450
Random	Variance	Std. Dev		
Plate	0.301	0.549		
Residual	1.182	1.087		

[a]Relative to females

4.5 Discussion

The present work reports for the first time sex-specific agglutination and lysis assays in Kentish plovers and Black-winged stilts. Despite the specific limitations of shorebird fieldwork (described in Székely et al., 2008; Székely, 2019), we obtained results consistent with similar prior immune analysis in other bird species (Matson et al., 2005; Hegemann et al., 2012; Hegemann et al., 2017). Although Hegemann et al. (2017) showed agglutination titres of 11 in non-breeding adult Ruffs (*Philomachus pugnax*), much higher than in both shorebirds here studied. It is interesting that most of male Black-winged stilt chicks had lysis titres close to zero (**Fig. 4.1**). This could either relate to young males of this species having low complement activity (Matson et al., 2005), or to a specific problem with the samples taken. But it is unlikely that only male samples were compromised given that all samples were handled in the same manner and we were blind to the sex of the chicks, even after determining the immune scores.

One alternative mechanism that could be associated with our findings is the development of immune tolerance, i.e. reduce the negative impact of an infection on the host fitness without directly affecting the pathogen burden (Medzhitov et al., 2012). Whether these birds choose to invest in costly immune defence to generate resistance or withstanding infection while paying a relatively low fitness cost is still to be investigated.

While our results showed that in adult Kentish plovers, males and females did not significantly differ in haemagglutination and haemolysis, recent studies show that these immune parameters seem to respond to specific variables in different fashion in males and females, rather than having mere sex differences in their titre levels. For instance, Pardal et al. (2018) found a negative correlation between colouration of breeding plumage and the immune parameters haptoglobin and haemolysis titres in males but not in females Black-tailed godwit (*Limosa limosa*), a long-distance migrant shorebird. Ndithia et al. (2019) found that titres of haemagglutination and haemolysis of males varied differently to the change of season (non-breeding period to

chick-feeding period) compared to those of females in Rufous-naped larks (*Mirafra africana*) and Red-capped larks (*Calandrella cinereal*). The latter study agrees with the results of Valdebenito et al. (in review), that showed that changes in males drove most of variations in sex biases in immunity in response to the transition from non-breeding to the breeding period in birds.

As our hypothesis was based on the ability of animals to recover from traumatic injuries (such as tissue damage), we cannot rule out possible sex differences in other immune parameters such as the antimicrobial activity test that could potentially provide different results because is more involved in wound repair (French & Neuman-Lee, 2012).

Black-winged stilts followed our expectations, showing no differences between the sexes and thus are in line with previous work in monogamous birds. However, a few other studies do show sex differences in monogamous bird species. Notably, Lee et al. (2006) found that male House sparrows (*Passer domesticus*) had stronger inflammatory response to phytohaemagglutinin (PHA) injection than females. Although House sparrows are monogamous birds, they present strong sexual dichromatism and males show intense aggressive behaviour towards other males (Summers-Smith, 1963). However, it is important to point out that PHA triggers an inflammatory and immune response in the host while we measured baseline immune function. Nevertheless, this suggests that perhaps variables other than mating system may be better predictors of intra-sexual agonistic behaviour in birds.

It is well-known that immune parameters may respond in different manner to different variables, and likewise in different species. For example, Palacios et al. (2018) found that cellular immunity was at its highest during breeding but humoral immunity peaked during moulting in Chinstrap penguins (*Pygoscelis antarcticus*). Although we did not find significant differences in either of the immune parameters, in both bird species the haemagglutination assays showed small sex differences. Detecting such small differences could be difficult with relatively small sample sizes because we risk incurring in type II error (Di Stefano, 2003). Future approaches should take this methodological limitation into consideration.

Body condition was included in the analysis of Kentish plovers to account for the effect of nutritional state because poor nutrition in animals could compromise immunity (e.g. Klasing, 2004; Hegemann et al., 2013; De Rosa et al., 2015; Childs et al., 2019). Energy is a limited resource, suggesting that animals should carefully allocate energy into traits that will maximise fitness (Sheldon & Verhulst, 1996). At the same time, nutrition has functional means in the organism because a minimal energy level is required to maintain baseline systemic functions such as reproduction or immune function (Hughes & Kelly, 2006; Rytter et al., 2014; Schorr & Miller, 2017). The latter should not affect our analysis because we studied breeding birds thus we could assume body condition was within the healthy range. Our results suggest that the immune function neither improve nor deteriorate in response of variations in body condition. Nilsson et al. (2007) found similar results in a study in Great tits (*Parus major*) that experimentally challenged birds with an antigen, yielding a small increase in

baseline metabolic rate compared to control birds, but the magnitude of the immune response of each individual did not match their increase in metabolic expenditure.

It has been shown that immunity of birds at hatching is not fully developed and it matures towards adulthood (Stambaugh et al., 2011). Although the body masses in Black-winged stilts chicks showed a relatively homogeneous gradient in development stage (**Fig. S4.2)** (Reed et al., 1999), this variable was not related to either natural antibodies or complement. Thus, our results suggest little variation in immune capacity in relation to developmental stage in chicks of this species, but our sample size was rather limited and adult birds were not considered as reference.

In conclusion, our results are encouraging and suggest that agglutination and lysis assays could provide reliable results for the study of innate immune function in shorebirds. This despite limitations of the field, particularly associated with high environmental temperatures and relatively long trapping times (compared with captures from nest boxes), that could alter the results of these two immune assays. On the other hand, our results do not support our predictions based on intra-sexual aggression. However, our experimental designed by no means would allow refute or validate such hypothesis. Also, the variety of mechanisms the immune system may rely on to effect its defensive function add important layers of complexity when attempting to draw conclusions. Moreover, mating systems is an important life history trait that influences many other variables in animals but could possibly lack consistency when predicting intra-sexual agonistic behaviour between species. Perhaps, augmenting the sample size could be of help since detecting small differences require higher statistical power. Also, testing this in a wider range of species could help ample the perspective of the underlying patterns. We believe that this topic (influence of mating system on immune defence) deserves more attention because, even though we did not find consistent support, mating system variation influences life histories of animals and thus thought to importantly relate with sex-specific mortality (Promislow et al., 1992; Owens & Bennett, 1994; Liker & Székely, 2005; Székely et al., 2014). Future studies addressing this question would benefit from including parameters that assess baseline immune function (e.g. bactericidal ability) and from experimentally measuring immune responses (e.g. PHA test, injection with lipopolysaccharide) in order to further detail mechanisms included in injury repair.

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4.7 Supplementary material

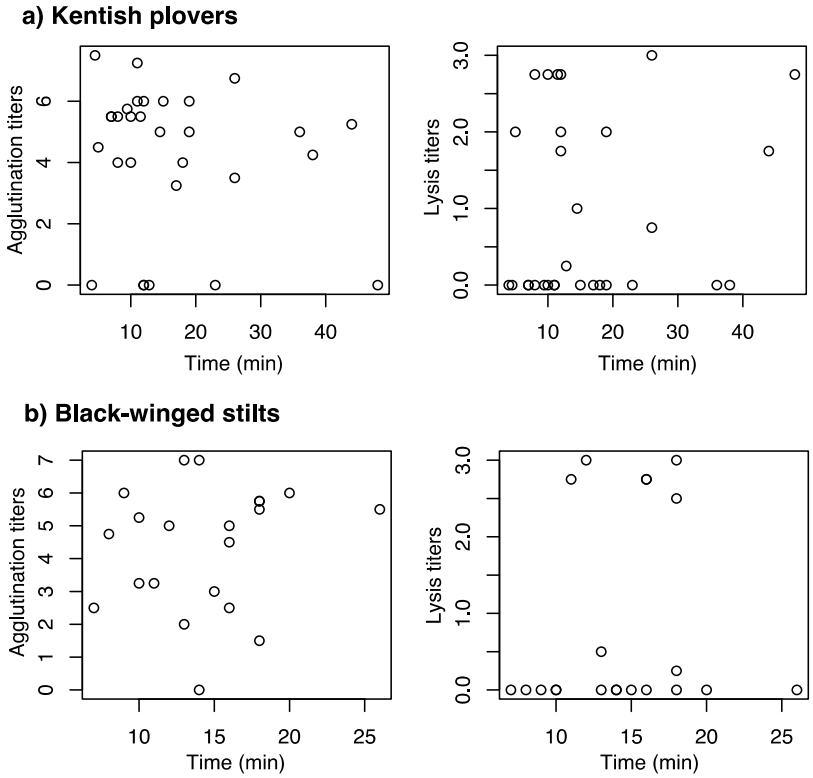

Figure S4.1 Capture-to-sampling time in relation to levels of agglutination and lysis titres in a) adult Kentish plovers and b) Back-winged stilt chicks.

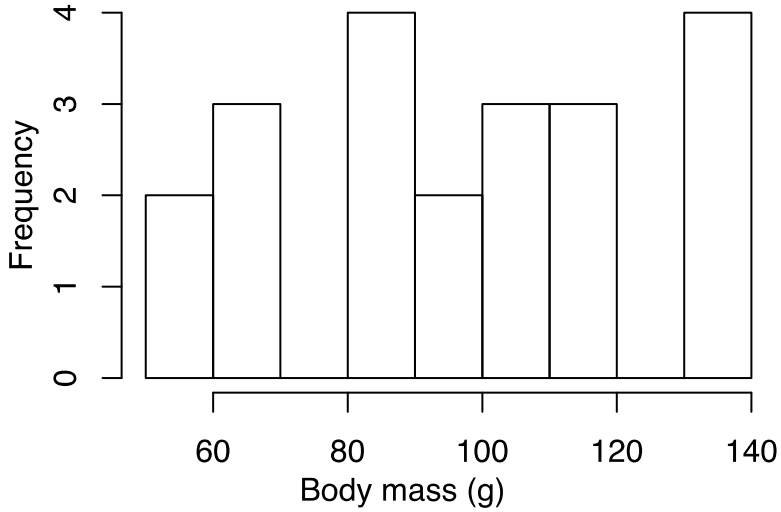

Figure S4.2 Frequency distribution of body mass in Black-winged stilt chicks.

Chapter 5 | Sex-specific brain immune gene expression in a small shorebird

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This paper reports on original research I conducted during the period of my Higher Degree by Research candidature

5.1 Abstract

Sex differences in morphological and behavioural traits in animals have been attributed to the Darwin-Bateman paradigm that proposes the emergence of costs and benefits specific to males and females, that often culminate in different mortality rates between the sexes. While the proximate causes of these sex differences in mortality may be diverse and frequently unknown, the immune system could directly influence individual survival because it is the main defence mechanism against pathogen infection, as well as maintaining a good cognitive ability (i.e. awareness of the surroundings) since could take part in predation risk in the wild. Here, we study the expression of immune genes from the brain in two populations of Kentish plovers (*Charadrius alexandrinus*), one from a coastal environment and the other from an extreme inland environment at high altitude, possibly exposed to difference pathogen pressures. Previous studies showed that Kentish plovers have female-biased mortality rate, thus we predicted that (i) males should present a higher number of immune system upregulated genes compared to females, and that (ii) immune gene expression should differ between coastal and inland breeders. Our results support the first expectation since immune gene expression was male-biased. However, we did not find expression difference in immune genes between coastal and inland habitats. Taken together, the sex different immune gene expression is consistent with expectations and thus match the direction of the known sex bias in mortality observed in many Kentish plover populations (female-biased). Nevertheless, more studies are needed to uncover the complex relationships within neuroimmunology and how it might influence sex-specific bird survival.

Keywords: sex-biased gene expression, avian immunity, RNA-Seq, brain, mating system

5.2 Introduction

Males and females often differ in numerous behavioural, morphological, and physiological aspects, which constitute a central theme in many scientific disciplines, including psychology (Eagly & Wood, 1999), medicine (Morrow, 2015), and biology (Andersson, 1994). The origin of these differences has been attributed to the Darwin-Bateman paradigm, which postulates that anisogamy imposes stronger sexual selection on males, which, in turn, drives the evolution of conventional sex roles in terms of female-biased parental care and male-biased sexual dimorphism. These sex differences may ultimately contribute to different mortality events between males and females, often observed in wild animals.

The nervous system coordinates an animal's actions and sensory information by transmitting signals to and from different parts of its body, with the brain being the organ that serves as its centre in all vertebrate animals. Sex differences in neurodegenerative diseases are well described in humans. For example, in Alzheimer disease, women show faster rates of brain atrophy than males (Ferretti et al. 2018). In non-human and wild animals this topic is significantly less explored, but evidence shows important sexual dimorphism in terms of brain gene expression (Yang et al., 2006; Naurin et al., 2011; Catalán et al., 2012; Sharma et al., 2014; Rotllant et al., 2017), although sex differences in immune components of the central nervous system are rarely mentioned.

The immune system is the main defence mechanism of an animal's organism against bacterial, viral and/or other microorganismal pathogens that can cause damage to the host. Bacterial and viral infections can be lethal, while other pathogens, like parasites, are better known for indirectly contributing to mortality by weakening the host and thus becoming more prone to predation (Genovart et al., 2010; Ingram et al., 2013; Adelman et al., 2017). In the nervous system, immune function seems to be under even stronger regulations since an insufficient response results in infection, whereas an excessive response results in prolonged inflammation and tissue damage (Kawli et al., 2013). Also, immune factors in the brain seem to serve a variety of non-immunological functions (Derecki et al., 2010; Radjavi et al., 2014). Many variables may influence immune response. Some of these include abiotic factors such as environmental variables that seem to indirectly modulate specific immune parameters of the host in response to environmental pressures exerted by pathogens and disease vectors that are expected to vary according to the given conditions of environmental productivity (Young et al., 1993; Pascual et al., 2002). For example, birds that inhabit in the tropics seem to upregulate aspects of their immune function in the wet season, presumably as defence mechanism against increased pathogen pressure that emerged from increased rainfall (Nwaogu et al., 2019; Tieleman et al., 2019). Sexual differences in immune defence are well described in birds, although, studies show substantial heterogeneity in their results. In a meta-analysis, Kelly et al. (2018) found that birds lacked overall sex differences in immunity when combining different age classes and immune parameters together, while a recent multi-species study showed that sex differences in immune defence in adult birds are more likely to appear during the breeding period (Valdebenito et al., in review). Thus, previous evidence shows that sex differences in immunity do exist

in nature, but predicting when such differences are likely to arise, as well as the direction of the skew, has proven challenging to anticipate.

The Kentish plover (*Charadrius alexandrinus*) is a small shorebird distributed along coastal and inland waterbodies across Europe, North Africa and Asia (del Hoyo et al., 2020). Kentish plovers have the particularity of presenting a variable mating system throughout its range which provides ideal circumstances to study mating system evolution (Székely, 2019). In general, mainland populations of Kentish plover are polyandrous and are characterised by presenting a balanced hatching sex ratio (close to 1:1) but a male-biased adult sex ratio, i.e. there are more males than females in the adult population (Eberhart-Phillips et al., 2018; Que et al., 2019). Sex-specific mortality is arguably the main cause of biases in adult sex ratio in birds (Székely et al., 2014b) and accordingly, Eberhart-Phillips et al. (2018) showed that both female-biased chick and adult mortality contributed substantially to the observed male bias in adult sex ratio in Kentish plovers from Turkey. Previous research addressing sex-specific parasite burden suggests that the answer may lay elsewhere as adult males and females show little to no difference in infection rates (Figuerola et al., 1996; Martínez-de la Puente et al., 2017; Valdebenito et al., 2020). On the other hand, aspects of the innate immunity also show little sex differences (Valdebenito et al., unpublished data). However, Valdebenito et al. (unpublished data) only evaluated the haemagglutination and haemolysis assays which represent a small proportion of the whole immune spectrum. In fact, this is the main caveat of "traditional" immune assays in ecology. In contrast, genomic methods provide a major advantage in this matter because it offers a broader scope by not only identifying genes involved in immune responses but also in important complementary processes such as inflammation.

In this study, we use information based on life history traits in order to predict possible sex differences in immunity of the brain in Kentish plover. We analyse transcriptome data produced in the work of Maher et al. (in preparation), consisting of sex-specific gene expression of brain tissue from two breeding populations of Kentish plover from two contrasting environments: one coastal and the other from the Tibetan plateau at 3,200 meters above sea level. From these data, we specifically selected genes involved in immune processes (according to gene ontology; GO) with the aim of evaluating sex differences in expression of immune genes, since sex differences in immunity could provide protection of the central nervous system and preserve cognitive ability (Ham & Lee, 2020), which in turn could benefit longevity.

Based on the bird's life history, i.e. sex-biased adult sex ratio and mortality, we expect males to have higher expression of immune related genes than females. Additionally, because the two populations studied were from different environments, we expected differences in immune gene expression between the environments based of possible differences in pathogen pressure.

5.3 Materials and methods

5.3.1 *Sampling*

Samples from 6 male and 6 female breeding Kentish plovers were collected from two populations with contrasting environments (**Fig. 5.1**): Bohai Bay at sea level (39° 7'6.22"N, 118°11'49.84"E; more details of the site are described in Que et al., 2015), and Qinghai Lake at 3,200 m above sea level (36°45'56.86"N, 100°43'21.68"E), in China. Fieldwork was conducted during the breeding period in May 2015, capturing incubating adults on the nest followed by morphometric measurements according to a standard protocol (see Székely et al., 2008). Lastly, to obtain blood and brain tissue, the birds were sacrificed conforming to the regulations of ethical conditions by the Chinese Animal Welfare Act (20090606), and of the Animal Welfare & Ethical Review Body of the University of Bath and the Institutional Ethical Committee of Animal Experimentation of Sun Yat-sen University (2005DKA21403-JK). This study did not involve endangered or protected species.

Tissue samples were collected for four brain regions: the hypothalamus, medial extended amygdala, nucleus accumbens and septum. Samples were dissected by first cutting the brain coronally using a stainless-steel rat brain matrix to standardise the rostrocaudal extent of the tissue slab sampled, and then individual regions were dissected by hand under a portable dissection microscope. Brain regions were identified on the basis of the chicken brain atlas (Puelles et al., 2018). The Kentish plover brain differs from the chicken, so anatomical landmarks visible in wet brain tissue were used to define the approximate borders of the dissections. The thickness of the slabs were cca. 1 mm. Brain tissue was stored in RNAlater (Qiagen). Samples were kept cold in the field using either liquid nitrogen or cold blocks, with samples transferred to standard freezers at the end of each day. Upon return from the field samples were stored at -80°C prior to RNA extraction. The hypothalamus sample of Female 1 from Bohai Bay was excluded from this study due to differences in tissue sampling in the field.

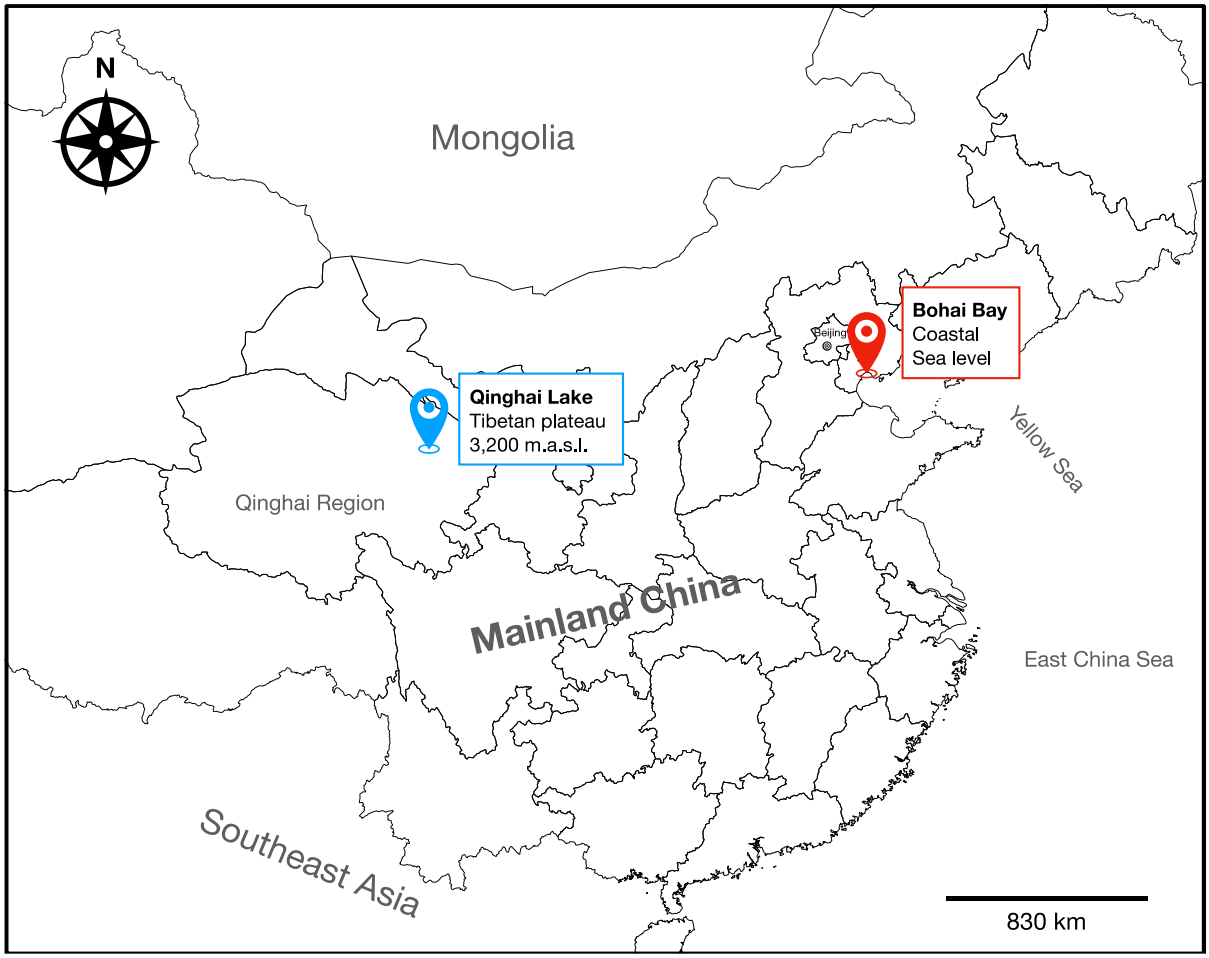

Figure 5.1 Sampling locations of Kentish plover in China. m.a.s.l. stands for meters above sea level.

5.3.2 *RNA extraction and next generation RNA sequencing*

RNA extraction and sequencing was performed by Novogene Beijing. 1% 202 agarose gels were used to monitor RNA degradation and contamination. The purity of the RNA was assessed using a NanoPhotometer spectrometer (IMPLEN, CA, USA). RNA concentration was then measured using a Qubit RNA Assay Kit in a Qubit 2.0 Flurometer (Life Technologies, CA, USA). RNA Nano 6000 Assay Kit with the Agilent Bioanalyzer 2100 system was used to assess RNA integrity. Sequencing libraries were prepared using 3µg RNA per sample and generated using NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB, USA), following manufacturer's recommendations with index codes added to allow identification for each sample. After cluster generation, which was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina), sequencing was performed on the library preparations on an Illumina HiSeq platform to generate paired-end reads with a fragment length of 300-500bp and an average paired end length of 150bp. Illumina sequencing resulted in 519,551,504 of raw paired-end reads sequenced in total for all samples.

5.3.3 *Transcriptome profile annotation*

Raw sequenced reads were cleaned using Novogene perl scripts. After cleaning, 506,421,928 of reads were of sufficient quality to align to the Kentish plover genome (Wang et al., 2019a). We discarded low quality and poly-N reads as well as reads containing adapters. All further analysis was performed on these high-quality reads. Cleaned reads were aligned to the Kentish plover genome using Tophat2 v2.0.14 (Kim et al., 2013) with an average insert size of 100 bp and 120 bp SD, and otherwise default parameters. Tophat outputs were cleaned using Samtools v1.2 (Li et al., 2009) to remove multi-mapped reads, unmapped reads and unmapped mates (-q 10, -F 12). A total of 253,809,249 reads were mapped in pairs to the Kentish plover genome. Raw read counts were extracted from cleaned Tophat outputs using HTSeq-count v0.6.1 (Anders et al., 2015).

5.3.4 *Differential expression analysis*

The analysis of differential gene expression was performed between the sexes and populations using *EdgeR* (Robinson et al., 2010) statistical package for R version 3.3.2 (R Core Team, 2016). All four brain tissue regions were pooled together for analysis. Clustering was performed using the "plotMDS" function (*EdgeR* package) after filtering to remove genes that had below 3 counts per million mapped reads (CPM) in at least 5 samples, and then normalising and calculating dispersions, further details below.

Differential expression analysis was performed on two different groupings of the dataset. The dataset was grouped together, first, regardless of population and then of sex, in order to calculate sex biases in gene expression and differences between environments (from Bohai Bay and Qinghai Lake), respectively. Genes were filtered and removed if they had below 3 CPM in at least 12 samples (11 samples for hypothalamus) for sex in the first set of analyses. For the next analyses, genes were filtered and removed if they had below 3 CPM in at least 6 samples (five samples, for hypothalamus analysis of female population-biased genes and analysis of Bohai Bay). Read counts were normalised using the trimmed mean of M-values (TMM). Dispersion was calculated using the "estimateDisp" function.

5.3.5 *Gene ontology term annotation and enrichment analyses*

Gene ontology (GO) enrichment analysis was performed on differentially expressed genes with a false discovery rate (FDR) <0.05. GO terms were obtained by blasting Kentish plover proteins against the RefSeq protein database using BLASTP v.3.2.0+, with an E-value of 1e-5 (Altschul et al., 1990). GO terms were then extracted using Blast2GO software v.4.1.9 (Götz et al., 2008) and then merged with GO terms obtained from InterProScan v.5.25 (parameters: -f xml, -goterms, -iprlookup) (Jones et al., 2014). GO categories were then split into groups associated with "biological processes", and then the subcategory "immune system process". GO categories with fewer than 50 genes were grouped into a single "small" category and genes without annotation were labelled in a "unannotated" category.

Enrichment analysis was conducted on up regulated and down regulated genes separately, with each up/down regulated gene counted for each category and comparing this to the expected number of genes per GO category. The expected number of genes per GO was calculated using 1,000 equally sized random samples.

The mean and the standard deviation were used to calculate Z-scores and *P*-values in order to establish significance and a Benjamini-Hochberg correction applied to adjust for multiple comparisons. Significant over-representation of GO terms was assumed if the adjusted *P*-value was <0.05. GO categories where the expected number of genes were less than one were only classified as significantly enriched if the observed number of genes was higher than one. GO enrichment analysis was based on methods used in Castillo-Morales et al. (2014).

5.3.6 *Sex-biased gene expression*

To compare if the distribution of male- or female-biased genes on the autosomes or Z chromosome were different than we would expect by chance we first assigned genes from the GFF file to chromosomes from the genome scaffold file using the "subsetByOverlaps" function in the *GenomicRanges* package in R (Lawrence et al., 2013). We then calculated 1,000 randomly selected gene sets of the same size as the number of male- and female-biased genes. Mean values and standard deviations for number of autosomal or Z chromosome genes expected to be found by chance were calculated and converted to Z scores. *P*-values were then calculated using the R function "pnorm" as a one-tailed test.

As this overrepresentation is likely to be the result of the fact that the avian Z chromosomes do not have an equivalent mechanism of X inactivation as that found in mammals, we re-assessed male-biased gene expression patterns on the Z chromosome correcting for the effect of dose by setting a log2FC cut off of ≥ 1. Although introducing this threshold reduced the number of male-biased Z-linked genes, the numbers were still higher than total female-biased genes. This had no effect on GO enrichment analysis, as we found no overrepresentation of any functional category for male-biased Z-linked gene either before or after correction.

5.3.7 *Sex- and population- specific gene expression*

To examine whether there was a difference in the proportion of sex- and population-biased genes, Chi-squared tests were used. We then looked at whether there was a difference between males and females in the proportion of sex-biased genes using Fishers exact tests. This was repeated but comparing the proportions of genes differentially expressed from Bohai Bay and Qinghai Lake. Finally, we examined whether the proportion of male- or female-biased genes found on the autosomes or Z chromosome were different between populations using Fishers exact tests.

5.4 Results

From a total of 14,892 genes expressed corresponding to 95% of Kentish plover annotated genes, we identified 403 genes associated to immune processes according to Gene Ontology biological process annotations (GO database http://geneontology.org/), corresponding to 2.71% of all protein coding genes.

Differential gene expression analyses identified 13 (3.26%) immune system sex-biased genes. From these, most exhibited male sex-biased expression with eleven male-biased genes *versus* only two genes were female-

biased (Fisher's exact test, *P*-value = 0.021, **Table 5.1**; **Fig. 5.2a**). Both female biased genes were involved in T-cell upregulation, consistent with a role in the activation of adaptive immunity. Male-biased genes are involved in the activation of various components of the immune system (details in **Table S5.1**).

Eight out of 11 male-biased genes were linked to the Z chromosome and one gene had an unknown chromosomal location. In the two female-biased genes, one was linked to the autosomes and the other belonged to an unknown chromosomal location (**Table S5.1**).

Comparing gene immune system gene expression profiles across the two environments, we found 25 (6.20%) highly expressed genes either in Bohai Bay or Qinghai Lake (**Fig. 5.2b**), but no differentially expressed genes were identified between the two environments (Fisher's exact test, *P*-value = 0.685). The majority of upregulated genes, however, were found in birds from Bohai Bay (**Table 5.1**).

Table 5.1 Biases in immune gene expression according to (a) sex and (b) habitat in brain tissue of Kentish plover (n = 6 males and 6 females) and results of Fisher's exact test.

a) Sex biases	Up males	Up females	*P*-value
Total of immune genes = 403	11	2	0.021
b) Habitat biases	Up Bohai Bay	Up Qinghai Lake	*P*-value
Total of immune genes = 403	14	11	0.685

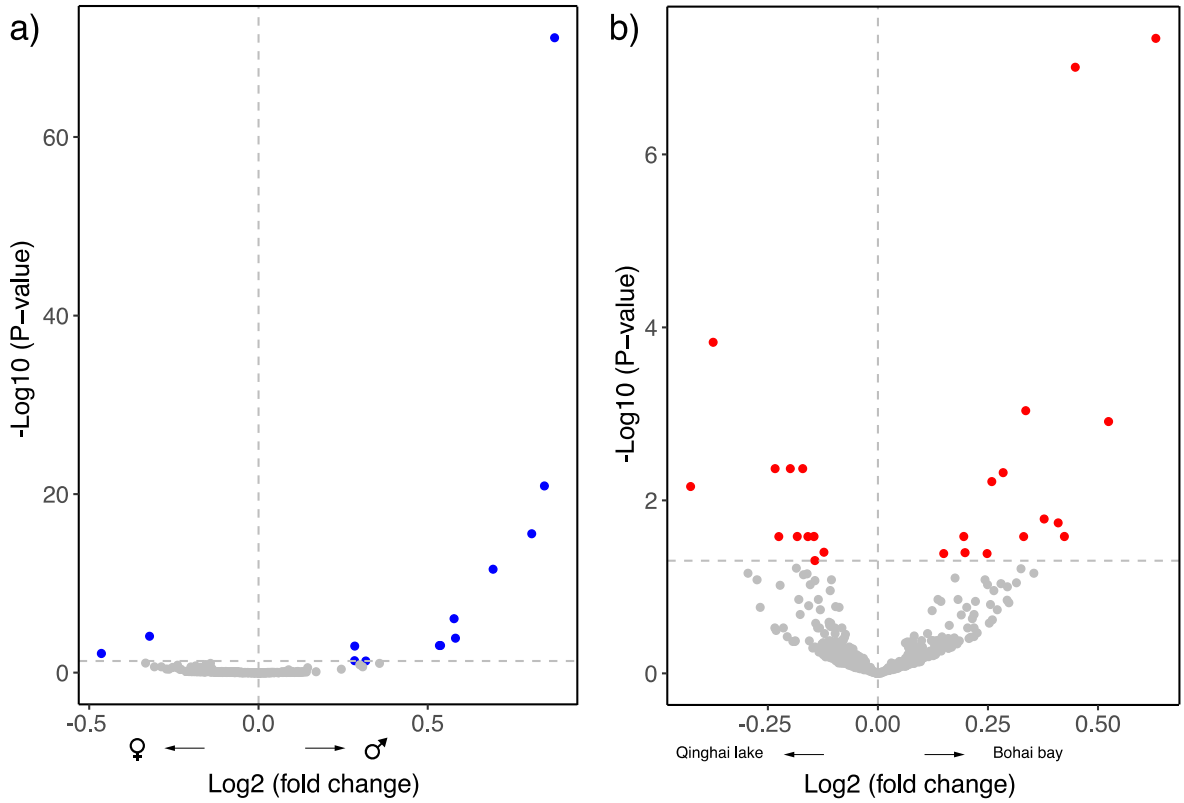

Figure 5.2 Biases in immune gene expression in brain tissue in Kentish plover. a) Shows sex-specific immune gene expression, and b) immune expression bias in relation to habitat. Genes with FDR >0.05 are coloured grey (below horizontal dashed line).

5.5 Discussion

In the present study we showed that, in Kentish plover, immune gene expression profiles from four brain areas differed between males and females, but no significant differences were identified between coastal and inland environments. Several recent studies have addressed aspects of immune defence using transcriptomic tools in birds (e.g.Videvall et al., 2015; Scalf et al., 2019; Videvall et al., 2020). This field is ripe for further expansion since despite the growing importance of sex-specific research across disciplines (Wilson, 2020), only Wang et al. (2019b) explored the impact of sex differences in captive Eurasian magpies (*Pica pica*), finding from blood samples important sex biases in expression of genes related to stress resistance, immunity, energy metabolism, reproduction, lifespan regulation, and diseases. Suggesting that females might be more susceptible to stress but have better immune defence than males, which contrasts our findings of males showing higher immune expression than females, although we did not evaluate expression of stress-related genes. However, to our knowledge, no previous study has investigated sex differences in expression of immune genes in the brain of wild birds, and we do acknowledge that tissue type determines profiles of gene expression (e.g. Watson et al., 2018), as well as noting that gene expression at the RNA level might not always correlate to actual protein levels (Mayfield et al., 2016), though several studies have shown good correlation between mRNA and protein concentrations (Lu et al., 2007; Li et al., 2014).

Causes of sex differences in mortality are source of constant expectations based on known sex differences in parasite burden, parental care, mating system, adult sex ratio (Moore & Wilson, 2002; Liker & Székely, 2005; Székely et al., 2014a). Because of the relevant role of the immune system in pathogen defence, it comes logical to propose strength of immune defence (or the lack of it) as another possible cause of mortality in the wild (Hegemann et al., 2013; Minias et al., 2018). However, not until recently, direct evidence for this associations were scarce. Froy et al. (2019) in a thorough study in a wild mammal showed for the first time that aspects of the immune system and its deterioration during natural aging accurately predicted overwinter survival in Soay sheep (*Ovis aries*). Although in birds, current research has not been able to reach such advanced point, our study modestly contributes in that direction. Previous studies in Kentish plover have shown that continental populations tend to have either sex biases in mortality or adult sex ratio (Lessells, 1984; Eberhart-Phillips et al., 2018). In the wild, most avian mortality events could be attributed to two broad causes: (i) mortality by disease and (ii) predation. The immune system should be of relevance in both instances because an animal needs to be in optimal physical condition (i.e. disease-free) in order to successfully scape from predators. Previous studies show that Kentish plover and allies have an apparent advantage in resisting blood parasites because of their consistent absence independently of the environment and vector abundance (Figuerola et al., 1996; Martínez-de la Puente et al., 2017). Interestingly, Valdebenito et al. (2020) and Halimubieke et al. (in preparation) found slim sex differences favouring females in cloacal prevalence of *Campylobacter*, *Salmonella*, and *Chlamydia*, and in cloacal bacteria diversity in Kentish plover, respectively. Although the immune-microbiome interaction is not yet well understood, perhaps female Kentish plovers have higher immune tolerance which allow them to have higher pathogen burdens without detrimental health effects (Medzhitov et al., 2012). Whether this is related to sex-specific survival is still unknown and requires further studies.

Despite rapid advances in genome sequence assembly and mapping tools, the exact location of many genes still remains unknown. We found that most male-biased genes were located on the Z chromosome which is in accordance with predictions of lack of complete dosage compensation in birds (Heard & Disteche, 2006; Itoh et al., 2007). However, the small number of sex-biased genes found prevents us from generating further conclusions.

Overall, our results show that the immune gene expression in the brain in the coastal environment is similar to the one inland in the Tibetan plateau. Two possible conclusions could be drawn from this. First, it may suggest that despite their extreme difference, the pathogen pressure in both environments were similar. Second, and more likely, the lack of differences between the environments may result simply because the birds only visit these sites to breed, consisting of a short 3-month window. Although the immune system of vertebrates has shown relatively quick adaptations to new environmental conditions (i.e. pathogen pressure) (Lindström et al., 2004; Peuß et al., 2020), a 3-month period over several generations is presumably too brief to generate considerable immune adaptations. Kentish plovers that breed in mainland China are believed to winter in the

southern limits of the country and Southeast Asia (MacKinnon et al., 2000), where they aggregate in large groups, sometimes with other shorebirds.

Despite the brain being a highly specialised tissue, we found comparable numbers of immune genes with previous studies. Wang et al. (2019b) found 395 genes related to immunity from blood in male and female Eurasian magpies, similar numbers to our findings that identified 403 immune genes. Although, it cannot be discarded that a proportion of the genes here detected belonged to blood imprisoned within the brain tissue, since the brain is among the organs with highest blood supply in the body. Also, it is known that some immune components serve non-immunological functions in the brain such as a role in memory of T lymphocytes in rats (Radjavi et al., 2014).

The use of genomic methods is a convenient approach for addressing a wide range of specific questions. However, it is necessary to employ careful interpretation because often single genes have many biological functions (**Table S5.1**). Here we showed that sex but not environment has an effect on immune gene expression in Kentish plover, although the latter needs further revisions. We believe that our results, although subtle, may represent the first approach into understanding proximate causes of sex-specific mortality. We are aware, however, that sex-specific mortality can be multifactorial and in order to determine whether sex differences in immune defence affect survival testing specifically for these associations is required. Further studies should emphasise on this approach.

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5.7 Supplementary material

Table S5.1 Genes upregulated by sex in Kentish plover and its function according to Gene Ontology (GO) in birds.

Gene ID	Sex upregulated	Chromosomal location	Main immune functions	Other biological functions
CHAAL00000003424	Females	Autosome	-Macrophage activation -T-cell differentiation in thymus	-Erythrocyte development
CHAAL00000008117	Females	Not assigned	-Regulation of T cell receptor signalling pathway -T-cell homeostasis	
CHAAL00000001967	Males	Autosome	-Activation-induced cell death of T cells -Negative regulation of apoptotic process -Positive regulation of apoptotic process -T-cell differentiation in thymus -T-cell proliferation involved in immune response -TOR signalling -Viral transcription	-Erythrocyte development -G1/S transition of mitotic cell cycle -Gastrulation -Glucose homeostasis -Mitotic cell cycle checkpoint -Mitotic nuclear division -Nuclear-transcribed mRNA catabolic process -Nonsense-mediated decay -Oogenesis stage -Ribosomal small subunit biogenesis -rRNA processing -Placenta development -Translational initiation -SRP-dependent co-translational protein targeting to membrane
CHAAL00000002488	Males	Z chromosome	-Positive regulation of lymphocyte proliferation -Thymus development	-Spleen development
CHAAL00000002733	Males	Z chromosome	-Immune response	-Regulation of transcription, DNA-templated -Transcription from RNA polymerase II promoter -Circadian rhythm
CHAAL00000006967	Males	Not assigned	-Fc-epsilon (IgE) receptor signalling pathway	
CHAAL00000007024	Males	Z chromosome	-Leukocyte tethering or rolling	
CHAAL00000007046	Males	Z chromosome	-Haematopoietic progenitor cell differentiation	
CHAAL00000008335	Males	Autosome	-Erythrocyte development	
CHAAL00000012274	Males	Z chromosome	-Innate immune response -Negative regulation of viral transcription -Positive regulation of NF-kappaB transcription factor activity	
CHAAL00000012457	Males	Z chromosome	-Epidermal cell differentiation -Negative regulation of cell proliferation	-Canonical Wnt signalilng pathway -Cellular response to growth factor stimulus

			-Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process -Negative regulation of heterotypic cell-cell adhesion -Negative regulation of interleukin-8 biosynthetic process -Negative regulation of NF-kappaB transcription factor activity -Negative regulation of response to cytokine stimulus -Positive regulation of nitric oxide biosynthetic process -Positive regulation of transcription regulatory region DNA binding -Stem cell population maintenance	-Cellular response to laminar fluid shear stress -Epidermis morphogenesis -Fat cell differentiation -Negative regulation of cell migration involved in sprouting angiogenesis -Negative regulation of chemokine (C-X-C motif) ligand 2 production -Negative regulation of transcription from RNA polymerase II promoter -Positive regulation of cellular protein metabolic process -Positive regulation of haemoglobin biosynthetic process -Positive regulation of telomerase activity -Positive regulation of transcription from RNA polymerase II promoter -Post-embryonic camera-type eye development -Post-embryonic haemopoiesis -Regulation of axon regeneration -Response to retinoic acid -Transcription from RNA polymerase II promoter
CHAAL00000012612	Males	Z chromosome	-B-cell homeostasis -B-cell proliferation -B-cell receptor signalling pathway -Germinal centre formation -Humoral immune response -Monocyte differentiation -Platelet formation -Positive regulation of B-cell proliferation -Regulation of germinal centre formation -Regulation of megakaryocyte differentiation	
CHAAL00000013158	Males	Z chromosome	-Antigen processing and presentation	

Chapter 6 | Seasonal variation in sex-specific immunity in wild birds

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This paper reports on original research I conducted during the period of my Higher Degree by Research candidature

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Seasonal variation in sex-specific immunity in wild birds

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Whilst the immune system often varies seasonally and exhibits differences between males and females, the general patterns in seasonality and sex differences across taxa have remained controversial. Birds are excellent model organisms to assess these patterns, because the immune system of many species is well characterised. We conducted a meta-analysis using 41 wild bird species from 24 avian families to investigate sex differences and seasonal (breeding/non-breeding) variations in immune status, including white blood cell counts, phytohaemagglutinin (PHA) test, bacteria-killing ability (BKA), haemolysis and haemagglutination assays. We found male-biased macrophage concentration, BKA and haemolysis titers, but only during the breeding season. Sex-specific heterophil concentrations, heterophil/lymphocyte ratios and PHA responses differed between breeding and non-breeding, suggesting larger changes in males than in females. Importantly, sex differences in immune status are stronger during the breeding period than during the non-breeding period. Taken together, our study suggests that both seasonal variation and sex differences in immune system are common in birds, although their associations are more complex than previously thought.

To thwart pathogens and keep infections at bay hosts rely on a competent immune system[1]. While the relationship between immune function and individual survival has been well documented[2–4], there has been relatively little research focused on sex differences in immune defence in free-living animals.

Differences in immune response between the sexes have been described extensively across vertebrates. These sex differences have been traditionally associated with the immunomodulating effect of sex hormones, where oestrogens, found in higher concentrations in females, act as weak immune-enhancers, and androgens, higher in males, as immune-suppressors[5,6]. However, these studies have been centred primarily on humans and laboratory animals, while there is increasing evidence suggesting that the association between sex hormones and sex differences in immunity in the wild are not as simple as first thought. Two independent meta-analysis showed that testosterone did not have a consistent overall immunosuppressive effect in males, and the effect depended on the taxa studied and whether the experimental manipulations involved hormone concentrations above physiological levels[7,8]. A recent study has also challenged the notion of sex biases in immunity by finding no overall sex difference in immune estimates in a large-scale comparative analysis including vertebrates and invertebrates[9]. However, Kelly et al.[9] showed that some patterns do arise when focusing on specific immune variables and taxonomic groups, such as mammals, which showed a strong male bias in specific pro-inflammatory cytokines. Kelly et al.[9] did not find overall sexual differences in birds immunity, but they concluded that future studies of sex differences in immunity should include variables known to affect immune functioning, such as age[10], nutritional state[11], photoperiod[12] or seasonality[13]. The latter variable is especially relevant, because seasonal changes, in particular the transition between the non-breeding and the breeding period, involve major physiological and behavioural changes. They may also include pronounced environmental shifts, particularly in species that migrate between breeding and non-breeding grounds, which is the case in many species of birds. Accordingly, several studies have found important sex-specific changes in immunity between the non-breeding and breeding period in birds. For example, Hõrak et al.[14] found that female Great Tits, *Parus major*, had more circulating lymphocytes than males in spring but not in summer. Merrill et al.[15] found that male Brown-headed Cowbirds, *Molothrus ater*, showed higher bactericidal capacity than females during the breeding period compared to the non-breeding period. Reasons behind such complex seasonal, species-specific and sex-specific immunity are not fully understood. Recurring explanations include sex-specific energetic and nutritional costs that may be traded

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off against immunity[16–18], thus resulting in an impaired immunity in the sex with higher energy expenditure (e.g. courtship displays, egg production, parental care[19–21]).

Alternatively, immune defence may be compromised in situations that cause strain or tension, i.e. stress[22]. Corticosterone, the main circulating glucocorticoid in birds, could play an important role here. First, because corticosterone is involved in regulating the metabolism[23], and second, as result of an increase in stress-induced corticosterone production (e.g. during territory defence) that could supress immune function[24–26]. However, a comprehensive analysis that simultaneously investigates seasonally-related and sex-specific immunity across bird species is largely lacking. Also, it is unknown whether potential sex-specific or seasonal patterns are consistent between immune parameters[27].

Here, in order to better understand the variation in avian immune function, we conducted a meta-analysis to test for seasonal (breeding versus non-breeding season) and sexual differences in immunity across bird species. Because of the known effects of ontogeny and captivity on immunity[28,29], we restricted our analysis to data from free-living adult birds. We included information from nine measurements characterising immune status: the relative frequency of four types of white blood cells (heterophils, lymphocytes, macrophages, eosinophils), the ratio of heterophils/lymphocytes (H/L ratio, a glucocorticoid-mediated immune index of stress), and four widely used immune response indexes (the phytohaemagglutinin test, bacteria-killing ability assay, haemolysis assay, and the haemagglutination assay). For each of these nine immune parameters we estimated their overall meta-analytic means (i.e. estimates of sex-specific immune biases). Based on previous studies[9,30], we expected no sex difference in white blood cells levels and a small female bias in the immune response indexes. Next, we broke down these overall estimates by season, and computed one estimate for the non-breeding period and one for the breeding period. This allowed us to test if these seasonal estimates were sex-biased, and if season, as a variable, had a significant effect on the immune parameters. Because breeding often incurs increased workload and higher energy demands compared to non-breeding birds in winter[16], we expected the two periods to differ from each other, and season to significantly affect immune variables[31,32].

Furthermore, we used the estimates from male and female individuals to test if the sexes could respond differently to the transition between seasons. Males are generally more involved in courting behaviour and intrasexual aggression; therefore, we predicted a possible stress-mediated immunosuppression[26] in males that could outweigh an alternative immunosuppression due to energetic trade-offs in females[21]. Thus, in the transition from non-breeding to breeding, males may exhibit stronger changes in immune estimates than females.

Materials and methods

Literature search. We systematically collected sex-specific white blood cells and immune response data from birds (PRISMA method[33]) using ISI Web of Science (see chart in Fig. S1; list of references in supplementary material). Our inclusion criteria required these data to be: (1) determined from adult birds with known sex, (2) obtained from free-living wild birds (not captive), and (3) from populations that were not experimentally manipulated. In order to conduct the meta-analytic calculations, the selected studies should provide the number of individuals examined per sex, the arithmetic mean of the immune variable measured and an estimate of its variance. We only included publications reporting results for both sexes to avoid difficulties generated by different sampling/diagnostic methods or different populations when calculating individual effect sizes.

Immune variables. *White blood cells (WBC).* We used data on the four most abundant WBC circulating in avian blood[34]: heterophils, lymphocytes, macrophages (also known as monocytes), and eosinophils. Basophils counts were discarded because of insufficient data available. The H/L ratio was also collected or calculated using the raw values of heterophils and lymphocytes. Elevated leucocyte number is a symptom of a stress syndrome, inflammatory processes and/or oxidative stress[35]. Usually, leucocytosis is caused by an elevated concentration of heterophils and/or lymphocytes[36,37]. Lymphocytes are immune cells that assist in the recognition and destruction of many types of pathogens. Although sometimes difficult to interpret, decreased lymphocyte concentrations may signal stress-induced immunosuppression[38], or may indicate a lack of parasite infections[39]. Heterophils are non-specific phagocytic cells that enter the tissues during inflammatory processes. Heterophil concentrations increase as a response to inflammatory processes, stress and infections[37]. Thus, the ratio of these two cell lines is considered a reliable proxy of physiological stress in birds[35,40]. Macrophages and eosinophils are less abundant in the avian blood than lymphocytes and heterophils. Their main function is to phagocytise and present antigens to T lymphocytes (T-cells), and to mediate the defence against parasite infections. Variation in their levels is commonly associated with pathogen infection[34]. WBC data came from apparently healthy animals (i.e. with no obvious signs of disease detected during handling), therefore assumed to represent baseline levels. The time between capture and sampling was not always available (details in Table S1), and Davis[41] showed that within one hour of capture the total leucocyte counts decreased as a result of handling stress, whereas proportions of each leucocyte type did not differ significantly. Therefore, we calculated WBC proportions (from the total number of leucocytes) to reduce between-study variation.

Estimates of immune response. We used four widely accepted measures of immune response in birds: the (1) phytohaemagglutinin test (PHA), that consists of a subcutaneous injection of this mitogen (phytohaemagglutinin) that triggers a local immune response mediated mostly by T-cell infiltration. Components of the innate and adaptive immune system take part in the response, which is estimated by measuring the degree of swelling of the skin, usually 24 h post-injection[42]. The (2) bacteria-killing ability assay (BKA) quantifies the ability of proteins in the plasma (such as complement, natural antibodies, and lysozymes) and/or phagocytic cells to kill bacteria[43]. The (3) haemolysis and (4) haemagglutination assays use foreign red blood cells (usually rabbit) to quantify titres of complement-like lytic enzymes (i.e. lysis, HL) and non-specific natural antibodies (i.e. agglutination, HA) in

plasma[44]. From each study we recorded whether the study was done during the breeding or the non-breeding season (hereafter season). Details of the breeding status extracted from each study are presented in Tables S1 and S2.

We used standard deviation (SD) as estimate of variance. When standard error was provided, we calculated SD using Eq. (1):

$$SD = SE \times \sqrt{n} \tag{1}$$

where SE is the standard error, and n is the sample size.

When 95% confidence intervals were given (in two studies), SD was calculated with Eq. (2):

$$SD = \frac{\sqrt{n} \times \left(upperCI - lowerCI\right)}{2\delta} \tag{2}$$

where n is the sample size, CI the confident intervals, and δ is the value for the t-distribution with degrees of freedom equal to the sample size minus 1 and a probability of 0.05[45].

Statistical analysis. *Phylogenetic meta-analysis.* To investigate sex biases in immunity, a phylogenetic multilevel meta-analysis was performed using the R package 'metafor'[46]. Effect sizes were computed using Hedge's g for standardised means because of its common use in ecology literature and for including a correction for small sample sizes[47,48]. Effect sizes are the standardised mean difference between two groups, which in our case corresponded to the mean of males relative to the female mean. Negative values of g indicate a female bias in the immune parameter studied and positive values a male bias. We conducted multilevel random-effect meta-analyses using the previously computed effect sizes as response variable and season (non-breeding/breeding) as moderator (i.e. fixed-effect). Phylogeny (a variance–covariance matrix) and study (to account for more than one species and/or immune estimate per study) were added as random-effect variables. We used the avian phylogeny proposed by Jetz et al.[49] and the analyses were conducted using consensus trees (one for each type of immune variable, Fig. S2) obtained by 50% majority-rule[50,51] from 1000 randomly selected trees from a pool of 10,000 available trees (http://birdtree.org) using the methodology described by Rubolini et al.[52]. These phylogenetic trees were not fully resolved, and polytomies were arbitrarily resolved by adding a branch distance of 10^{-8} to one randomly chosen branch in the polytomy using the function 'multi2di' from the R package 'ape'[53]. Publication bias (due to missing studies that were not published because of negative or null results[54]) was evaluated by inspecting the symmetry in funnel plots and using the Egger's regression test[55,56] by including the standard error of the effect sizes as an additional moderator within the model. If the intercept significantly deviated from zero (significance of $p < 0.10$[55]), the overall relationship between the precision and size of studies included in the data set was considered asymmetrical or, in other words, biased[56]. Of the nine fitted models, only macrophages and eosinophils suggested presence of publication bias (both $p < 0.001$). Diagnostic tests for identifying influential data points and outliers, and rules for excluding these types of cases are not well established, particularly for multivariate/multilevel meta-analytical models[57]. We used the approach described by Habeck and Schultz[58] by identifying the influential outliers causing the bias and running the models after excluding these values. We report results after removing one effect size from the final model of macrophages, and two from the model of eosinophils (see Table S3 for the final sample sizes used in the analyses). The effect of season on the immune sex-bias was tested using the Omnibus test (QM) for moderators (a Wald-type Chi-squared) implemented within the function 'rma.mv' (metafor R package), which tests whether the explained heterogeneity by a parameter (here, season) is significantly greater than the unexplained overall heterogeneity[46]. The HL and HA assays were excluded from further analysis because only estimates of breeding birds were available. We used Cochran's Q test to estimate whether the (residual) heterogeneity among effect sizes was greater than expected by sampling error alone[59]. We also calculated the variance in effect sizes due to phylogenetic relatedness ($I^2_{phylogeny}$), differences among studies (I^2_{study}), and the total variance attributed to the random effect variables (i.e. the addition of the two effects, I^2_{total}).

Generalised linear mixed models. To explore if seasonal changes affected the sexes independently, we fitted generalised linear mixed models by Markov chain Monte Carlo techniques using the R package 'MCMCglmm'[60]. This analysis differs from the previous in that here we analysed variation of each sex parameters according to season, instead of one 'combined' effect size. This approach helps to understand how each sex responds to season, because changes in effect size estimates from the non-breeding period to the breeding period may be the result of increases or reduction in one or both sexes at once. Each of these seven models (HA and HL were excluded) had immune variables as response variable, and season, sex (females/males), and the two-way interaction of season and sex as explanatory (fixed-effect) variables. All models included study and phylogeny as random-effect variables. The H/L ratio was log-transformed. The H/L ratio and PHA models were run with a Gaussian family distribution. The rest of the models were run using a binomial family distribution. To investigate whether the above comparisons may have been confounded by different species composition in the breeding and non-breeding samples, we ran these models two times. First using the full dataset, and then using a subset of the data that included only those species for which we had data from both non-breeding and breeding seasons (Table S4). We used parameter expanded (random-effects) and inverse-Wishart priors (fixed-effects) based on improving model convergence. Further details of model specification are given in the supplementary material. Convergence and autocorrelation levels were assessed through the Gelman-Rubin test[61], trace graphs and the 'autocorr' function, implemented in the R package 'coda'[62]. MCMCglmm results are expressed as posterior mean, lower and upper 95% credible intervals, and significance as a pMCMC value.

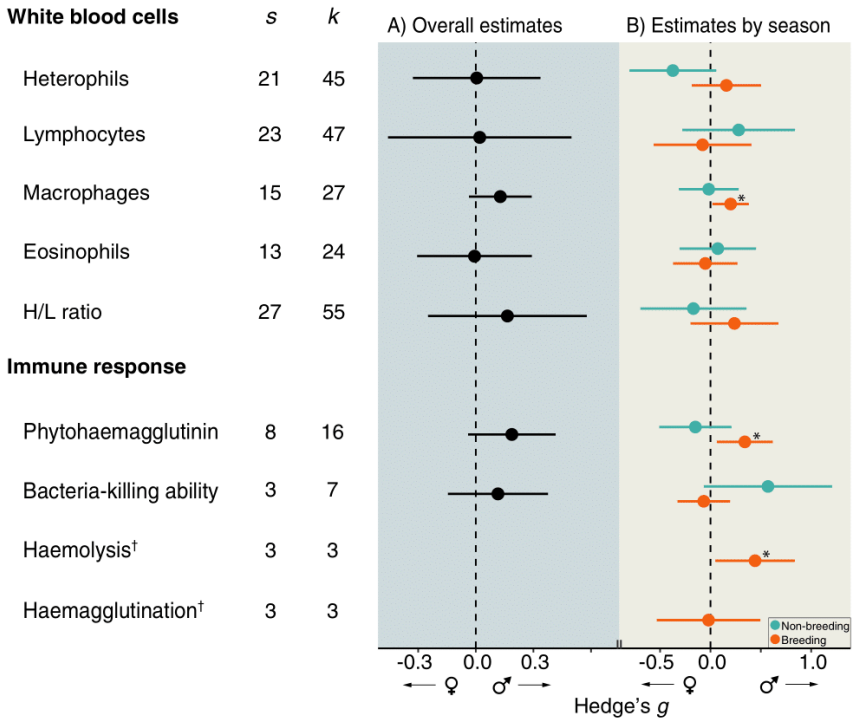

Figure 1. Sex bias in white blood cells and immune response assays in adult wild birds (weighted average effect sizes and 95% confidence intervals). (**A**) Overall in immune estimates. (**B**) Immune estimates for non-breeding (in cyan) and breeding (in orange) birds. Weighted averages were tested whether they differed significantly from zero (i.e. no sex bias, dashed line; see statistics in Table 1), where positive estimates mean male bias and negative female bias. *s*, number of species; *k*, number of effect sizes; H/L ratio, heterophils/lymphocytes ratio; *statistical significance ($p < 0.05$); †data from breeding birds only.

Results

Sex biases in immunity and the effect of season (meta-analysis). Our results show that across all immune variables, while there was no overall difference between males and females (Fig. 1A), there was an important variation in sex differences between the non-breeding and the breeding period (Fig. 1B; Table 1). Macrophage concentration, haemolysis score and PHA response were significantly male-biased during breeding (Fig. 1B). During the non-breeding period, BKA tended to be higher in males ($p = 0.089$) while heterophil concentration tended to be higher in females ($p = 0.079$). Both phylogeny and study explained an important proportion of the variance in immune variables (Table 1).

Seasonal changes had a significant effect on the sex bias estimates of three immune parameters: heterophil concentration, H/L ratio and PHA response (Omnibus test of coefficients [df = 1]: 8.131, $p = 0.004$; 8.547, $p = 0.003$; 4.832, $p = 0.028$, respectively; Table 2). These results indicate that, in these immune parameters, the immune estimates from the non-breeding and breeding periods were significantly different from each other. In all cases the direction of the skew was towards males. A non-significant trend in the opposite direction was found for lymphocyte concentration and for BKA, where estimates obtained in the breeding season deviated towards females (Table 2).

Effect of seasonal changes on males and females (GLMM analysis). The GLMM–MCMC models revealed a significant interaction between season and sex for heterophil concentration and H/L ratio, indicating that these variables show a greater change between non-breeding and breeding season in males than in females (Fig. 2A,E; Table 3). These results were consistent between models using the whole data set and those using a subset of species for which data during both the non-breeding and breeding season were available (Table S5). Also, for BKA, seasonal changes tended to differ between males and females when tested with the full data set ($p = 0.078$), but the pattern became weaker when using the subset of data (Table S5), arguably due to low sample size in this variable (Fig. 2G). The other immune parameters (lymphocytes, macrophages, eosinophils, PHA) showed no significant sex differences in the change between non-breeding and the breeding period, suggesting that males and females either increase or decrease their levels in comparable proportions (Fig. 2, Table 3).

Immune variable	$I^2_{phylogeny}$ (%)	I^2_{study} (%)	I^2_{total} (%)	Q_{REML} (*P*)	Overall estimates		Estimates by season			
					Overall (95% CI)	*Z* statistic (*P*)	Non-breeding (95% CI)	*Z* statistic (*P*)	Breeding (95% CI)	*Z* statistic (*P*)
(a) White blood cells										
Heterophils	23.69	46.08	69.76	171.238 (<0.001)	0.005 (−0.327, 0.337)	0.027 (0.978)	−0.373 (−0.804, 0.057)	−1.698 (0.089)	0.158 (−0.186, 0.502)	0.902 (0.367)
Lymphocytes	45.17	33.91	79.07	182.957 (<0.001)	0.020 (−0.457, 0.498)	0.084 (0.933)	0.280 (−0.279, 0.839)	0.981 (0.327)	−0.079 (−0.564, 0.406)	−0.318 (0.750)
Macrophages	<0.01	22.98	22.98	26.780 (0.367)	0.128 (−0.036, 0.291)	1.531 (0.126)	−0.018 (−0.314, 0.279)	−0.117 (0.907)	0.200 (0.020, 0.380)	**2.175 (0.030)**
Eosinophils	38.52	0.00	38.52	26.520 (0.277)	−0.007 (−0.305, 0.292)	−0.045 (0.964)	0.073 (−0.307, 0.452)	0.375 (0.708)	−0.052 (−0.371, 0.268)	−0.317 (0.752)
H/L ratio	40.18	33.39	73.58	191.669 (<0.001)	0.143 (−0.296, 0.582)	0.639 (0.523)	−0.171 (−0.700, 0.358)	−0.634 (0.526)	0.240 (−0.199, 0.680)	1.071 (0.284)
(b) Immune response										
PHA	0.00	9.54	9.54	13.839 (0.462)	0.188 (−0.040, 0.415)	1.614 (0.107)	−0.150 (−0.508, 0.208)	−0.821 (0.412)	0.341 (0.063, 0.619)	**2.407 (0.016)**
BKA	<0.01	0.00	<0.01	15.122 (0.010)	0.115 (−0.145, 0.376)	0.868 (0.385)	0.571 (−0.066, 1.207)	1.758 (0.079)	−0.067 (−0.328, 0.194)	−0.503 (0.615)
HL	33.39	0.00	33.39	2.080 (0.354)	–	–	–	–	0.443 (0.048, 0.837)	**2.199 (0.028)**
HA	28.45	33.48	61.93	4.605 (0.100)	–	–	–	–	−0.019 (−0.533, 0.495)	−0.074 (0.941)

Table 1. Sex bias in (a) white blood cell types and the H/L ratio, and (b) immune response assays in adult wild birds. *p* values < 0.05 in bold. H/L ratio, heterophils/lymphocytes ratio; PHA, phytohaemagglutinin test; BKA, bacteria-killing ability assay; HL, haemolysis assay; HA, haemagglutination assay; $I^2_{phylogeny}$, variance due to phylogenetic relatedness; I^2_{study}, variance due to differences among studies; I^2_{total}, total variance attributed to the random effect; Q_{REML}, Cochran's *Q* test for (residual) heterogeneity. *Z* statistic tests if immune parameter estimate differ from zero (no sex difference).

Immune variable	*QM* (df = 1)	*P*
White blood cells		
Heterophils	8.131	**0.004**
Lymphocytes	3.453	0.063
Macrophages	1.662	0.197
Eosinophils	0.488	0.485
H/L ratio	8.547	**0.018**
Immune response		
PHA	4.832	**0.028**
BKA	3.301	0.069

Table 2. Omnibus test of coefficients (*QM*) testing for the effect of season on the sex bias of the immune parameters studied. *p* values < 0.05 in bold. H/L ratio, heterophils/lymphocytes ratio; PHA, phytohaemagglutinin test; BKA, bacteria-killing ability assay.

Discussion

To our knowledge, this is the first multi-species analysis investigating the effect of seasonal variation on sex-specific immunity in wild birds. We showed an overall lack of sex differences in the immune variables studied. However, when taking season into account, subtle but consistent patterns arise indicating that males are undergoing more substantial reorganization of their leukocyte composition during reproduction than females.

Similar to Kelly et al.[9], the overall meta-analysis of the immune parameters showed no significant sex biases in immunity, although with subtle variations of male and female biases in the estimates. In multilevel meta-analysis, non-significant results could originate from small effect sizes being close to zero (i.e. no sex difference). However, the heterogeneity attributed to random effect variables was rather high (I^2 and *Q* test[63]), suggesting that our data set had great variation of opposing effect sizes (i.e. some species estimates showing a male bias and others a female bias). Breaking the immune estimates down by season revealed notable sex differences between the non-breeding and breeding period, with macrophage concentration, PHA response and haemolysis score being male-biased, and a significant seasonal influence on the estimated sex bias for heterophil concentration, H/L ratio and PHA response. Heterophils and lymphocytes make up to 95% of the total leucocyte count[64]. Both cell types have important roles in innate immunity, but only lymphocytes participate in adaptive immunity[34,36]. Macrophage levels were male-biased during the breeding period, but no sex differences were found for levels of eosinophils. Macrophages and eosinophils are specialised against unspecific cells like apoptotic cells or microbes,

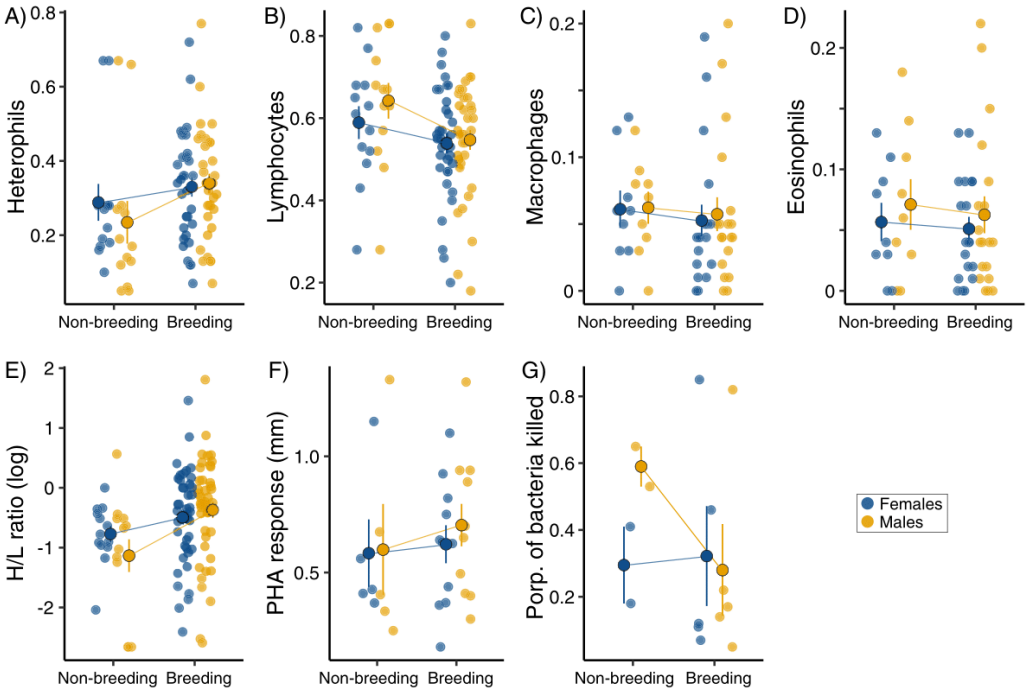

Figure 2. Characteristics of the immune system in wild birds. White blood cells (**A**–**D**, expressed as the proportion of the total white blood cell count), heterophils/lymphocytes (H/L) ratio (**E**), phytohaemagglutinin response (**F**), and bacteria-killing ability (**G**) in breeding and non-breeding birds (blue and yellow dots refer to females and males, respectively). Large outlined dots and whiskers are arithmetic means and standard errors, respectively.

and against parasite infections, respectively[1]. Studies reporting sex differences in these two leucocyte lines in birds are scarce. Variation in levels of eosinophils are attributed to different levels of infection by gastrointestinal parasites in birds[65,66], and sex differences in macrophage gene expression associated to the sex chromosomes have been reported in chicken[67].

Seasonally varying levels in (1) stress (defined as a physiological response due to strain or tension), (2) hormones and (3) workload may form the basis of mechanisms that could explain our findings. First, stressors associated with breeding could cause immunosuppression. It has been suggested that behaviours such as sexual display and nestling feeding in birds are comparable to strenuous exercise in that they impose a high metabolic rate[68,69]. In addition, because males are in general more aggressive and dominant than females, in periods of low food abundance (such as winter) males could secure their access to food over females, which seems to cause strain in birds[70–72]. This could explain our results for H/L ratio, since increments in H/L ratio appear to be associated with sustained stress in birds[35,40,73]. Although the H/L ratio was not sex-biased during the non-breeding or the breeding season, both estimates were different from each other, and males experienced a greater change between the seasons than females. Second, the breeding period in birds is characterised by behavioural changes triggered by the sex hormones. Androgens and oestrogens have traditionally been thought to influence immunity in birds by up- or down-regulating their immune system. However, current evidence disregards sex hormones (mostly testosterone) as important immune modulators in birds[7,8]. For instance, Roberts et al.[74] found no effect of testosterone on immune response in Japanese Quail, *Coturnix japonica*. Li et al.[75] found that in Eurasian Tree Sparrow, *Passer montanus*, testosterone concentration was positively correlated with the strength of PHA response in males, whereas in females the correlation was negative. Additionally, Duffy et al.[76] concluded that the increase in plasma corticosterone upon treatment with testosterone implants in European Starlings, *Sturnus vulgaris*, was the likely cause of immunosuppression in males and females rather than testosterone itself. Conclusions from studies in wild birds have been based mainly on correlational observations, which may obscure the real effect of sex hormones on immunity. Furthermore, our results are consistent with previous literature failing to find consistent support for the immunocompetence-handicap hypothesis[7,9,77,78].

Third, reproduction requires temporarily elevated energy and nutrient input, which could compromise immune function[16,79,80]. Trade-offs between reproduction and self-maintenance may vary both between the sexes and over specific stages of reproduction while each sex invests in traits that will maximise reproductive success[81]. Accordingly, but depending on breeding system and sex roles, during mating it might be the males but during egg production and incubation the females that compromise their immune function relatively more. For example, in a clutch size manipulation experiment in Common Eiders, *Somateria mollissima*, Hanssen et al.[82] showed that females incubating larger clutches lost more body mass and showed reduced immune function

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
(a) Heterophils (n = 90, s = 21)				
Intercept	−0.448	−1.336	0.338	0.236
Season (breeding)[a]	−0.113	−0.579	0.317	0.602
Sex (males)[b]	−0.396	−0.723	−0.124	**0.012**
Season (breeding)[a] * sex (males)[b]	0.429	0.036	0.760	**0.018**
Random				
Study	0.560	0.070	1.069	
Phylogeny	0.411	< 0.001	1.310	
Residual	0.081	0.033	0.136	
(b) Lymphocytes (n = 94, s = 23)				
Intercept	−0.091	−0.768	0.548	0.782
Season (breeding)[a]	0.035	−0.405	0.393	0.864
Sex (males)[b]	0.254	−0.026	0.580	0.094
Season (breeding)[a] * sex (males)[b]	−0.221	−0.570	0.148	0.238
Random				
Study	0.170	< 0.001	0.426	
Phylogeny	0.336	< 0.001	0.840	
Residual	0.109	0.052	0.163	
(c) Macrophages (n = 56, s = 15)				
Intercept	−3.494	−5.220	−1.857	**0.002**
Season (breeding)[a]	−0.476	−1.026	0.151	0.112
Sex (males)[b]	0.019	−0.375	0.411	0.932
Season (breeding)[a] * sex (males)[b]	0.071	−0.485	0.494	0.750
Random				
Study	1.065	< 0.001	3.393	
Phylogeny	1.839	< 0.001	5.211	
Residual	0.008	< 0.001	0.029	
(d) Eosinophils (n = 56, s = 13)				
Intercept	−3.873	−5.978	−2.199	**0.002**
Season (breeding)[a]	0.220	−0.425	0.793	0.448
Sex (males)[b]	0.251	−0.187	0.741	0.256
Season (breeding)[a] * sex (males)[b]	−0.042	−0.620	0.491	0.878
Random				
Study	1.483	0.140	3.671	
Phylogeny	2.548	0.422	5.853	
Residual	0.043	< 0.001	0.134	
(e) H/L ratio (n = 110, s = 27)				
Intercept	−0.577	−1.200	0.096	0.088
Season (breeding)[a]	0.054	−0.277	0.422	0.764
Sex (males)[b]	−0.361	−0.677	−0.006	**0.032**
Season (breeding)[a] * sex (males)[b]	0.483	0.095	0.875	**0.014**
Random				
Study	0.517	0.137	0.973	
Phylogeny	0.233	< 0.001	0.777	
Residual	0.182	0.130	0.237	
(f) PHA response (n = 32, s = 8)				
Intercept	0.648	0.182	1.139	**0.012**
Season (breeding)[a]	0.097	−0.140	0.320	0.420
Sex (males)[b]	0.017	−0.219	0.240	0.884
Season (breeding)[a] * sex (males)[b]	0.060	−0.239	0.309	0.664
Random				
Study	0.050	< 0.001	0.231	
Phylogeny	0.155	< 0.001	0.424	
Residual	0.034	0.018	0.056	
(g) BKA assay (n = 14, s = 3)				

Continued

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*
Intercept	−0.253	−4.987	4.873	0.864
Season (breeding)[a]	−0.195	−1.709	1.318	0.718
Sex (males)[b]	1.356	0.011	2.889	0.056
Season (breeding)[a] * sex (males)[b]	−1.594	−3.499	0.219	0.078
Random				
Study	6.441	< 0.001	22.95	
Phylogeny	5.375	< 0.001	20.10	
Residual	0.598	0.066	1.510	

Table 3. White blood cell levels and immune response in wild birds in relation to sex and season (MCMC generalised linear mixed models; *n*, total number of individuals; *s*, number of species). *p* values < 0.05 in bold. [a]Relative to the non-breeding period. [b]Relative to females.

(lymphocyte levels and specific antibody response). While in lekking males of Greater Sage-grouse, *Centrocercus urophasianus*, alfa males showed a daily energy expenditure two times higher than a non-displaying male and four times higher than their basal metabolic rate[68]. Unfortunately, the data collected for our meta-analysis were obtained from studies that sampled at various moments throughout the entire breeding period and from species with different breeding systems, which prevented us from drawing further conclusions. Likewise, the present analysis relied on a selection of more generic indicators of (innate) immunity, and future research will profit by including also more specific indicators and those that belong to the adaptive arm of the immune system. Moreover, immune tolerance and autoimmunity can significantly influence the cost balance and, therefore, the outcome of reproduction-immunity trade-offs[83,84].

Although data on immune response variables were not available for many species, we did find differences between males and females. The four immune assays analysed reflect innate immunity, except the PHA test that, if repeated more than once, also includes components of the adaptive immunity[85]. The PHA test and the haemolysis assay were significantly male-biased during breeding, although the latter estimate was obtained only from three effect sizes. Generally, the PHA response in birds appears to decrease during breeding[42,86], although no association with breeding was found in Chinstrap Penguins, *Pygoscelis antarctica*[27]. In Eurasian Tree Sparrow, Li et al.[75] found no differences in PHA responses between breeding males and females, while Zhao et al.[87] found that body condition but not breeding stage correlated with their haemolysis levels. Interestingly, in our analysis the PHA test and the BKA assay showed opposite responses to season (Figs. 1B and 2F,G). In both cases the differences seemed to be largely driven by changes in males (Fig. 2F,G). However, with a relatively small sample size and considering the subset analysis, the results of the model interaction of BKA assay should be taken cautiously. Yet another possible explanation for our results on immune response variables might be based on sexual selection theory, and predicts that the competing sex (males in most mating systems) will evolve higher innate immune response. According to this scenario, selection would favour strong inflammation responses as an aid for healing wounds, because the competing sex is more involved in aggressive interactions causing physical injury[88,89]. The inclusion of mating system should thus be considered in future studies in order to test this hypothesis.

Here we have shown that across wild birds, sex differences in certain measures of immune status and response associated to the breeding season may occur. The exact causes of these seasonal patterns of sexual changes in immune function are difficult to identify. In addition to the complex nature of the avian immune system, a number of unaccounted variables could directly or indirectly confound our analysis, such as genetic, environmental and ecological factors (like photoperiod or mate competition), with the potential of affecting one or several immune components, and in different sex-specific fashion. The scarcity of available studies to date prevented us also from exploring factors like mating system and parental care, which seem important to further understand the causes of seasonal and sexual changes in immunity. Nonetheless, our results highlight sexual differences in immune function as a relevant topic that requires further attention in wild birds.

Data availability

The full dataset and R code can be found at https://doi.org/10.6084/m9.figshare.13476819.v1.

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Author contributions
J.O.V. collected the data, conducted the data analysis and wrote the paper. All authors contributed substantially to study design and revisions of the paper.

Competing interests
The authors declare no competing interests.

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6.1 Supplementary material

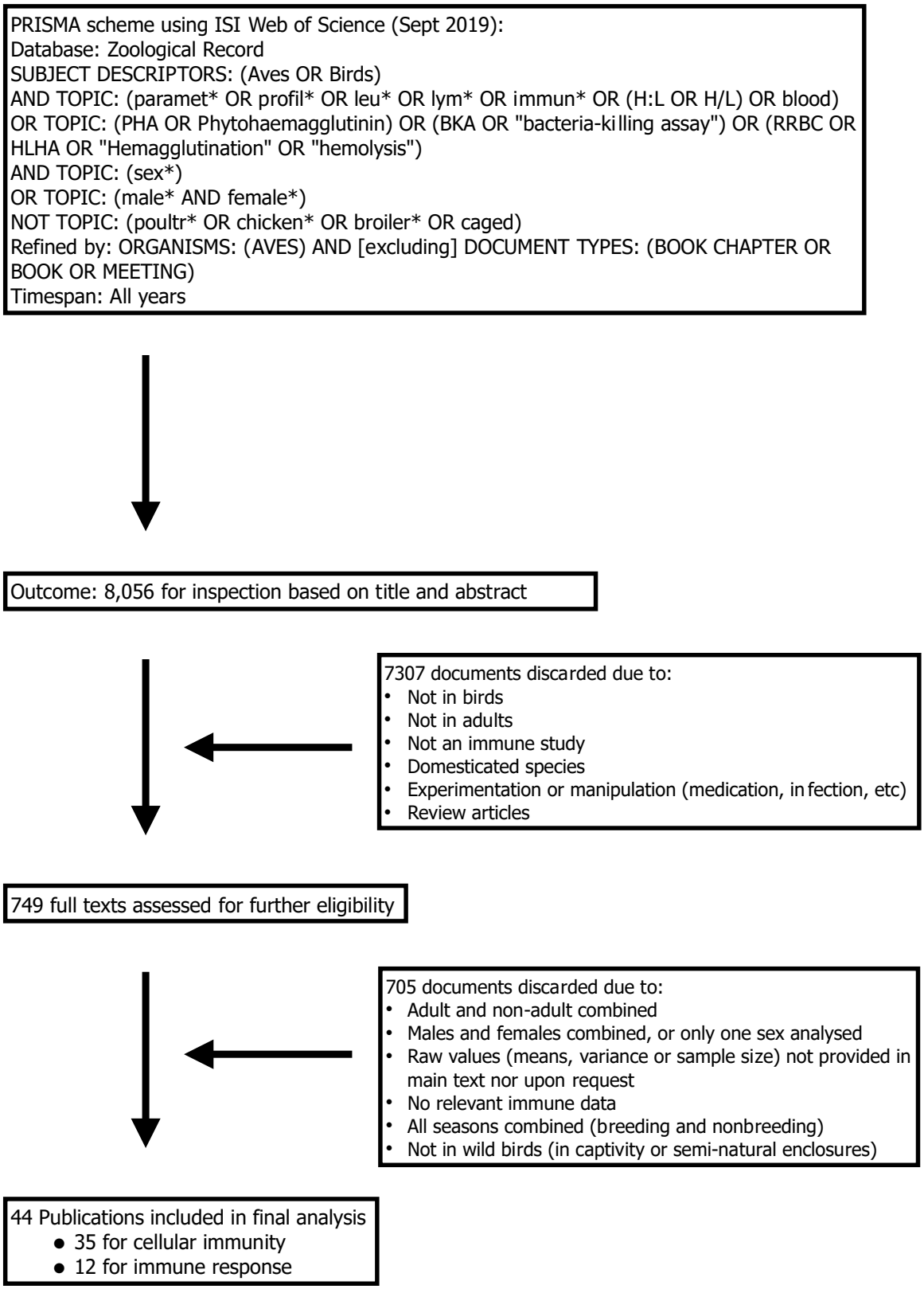

Figure S6.1 Searching parameters and PRISMA scheme followed to obtain publications used in analysis.

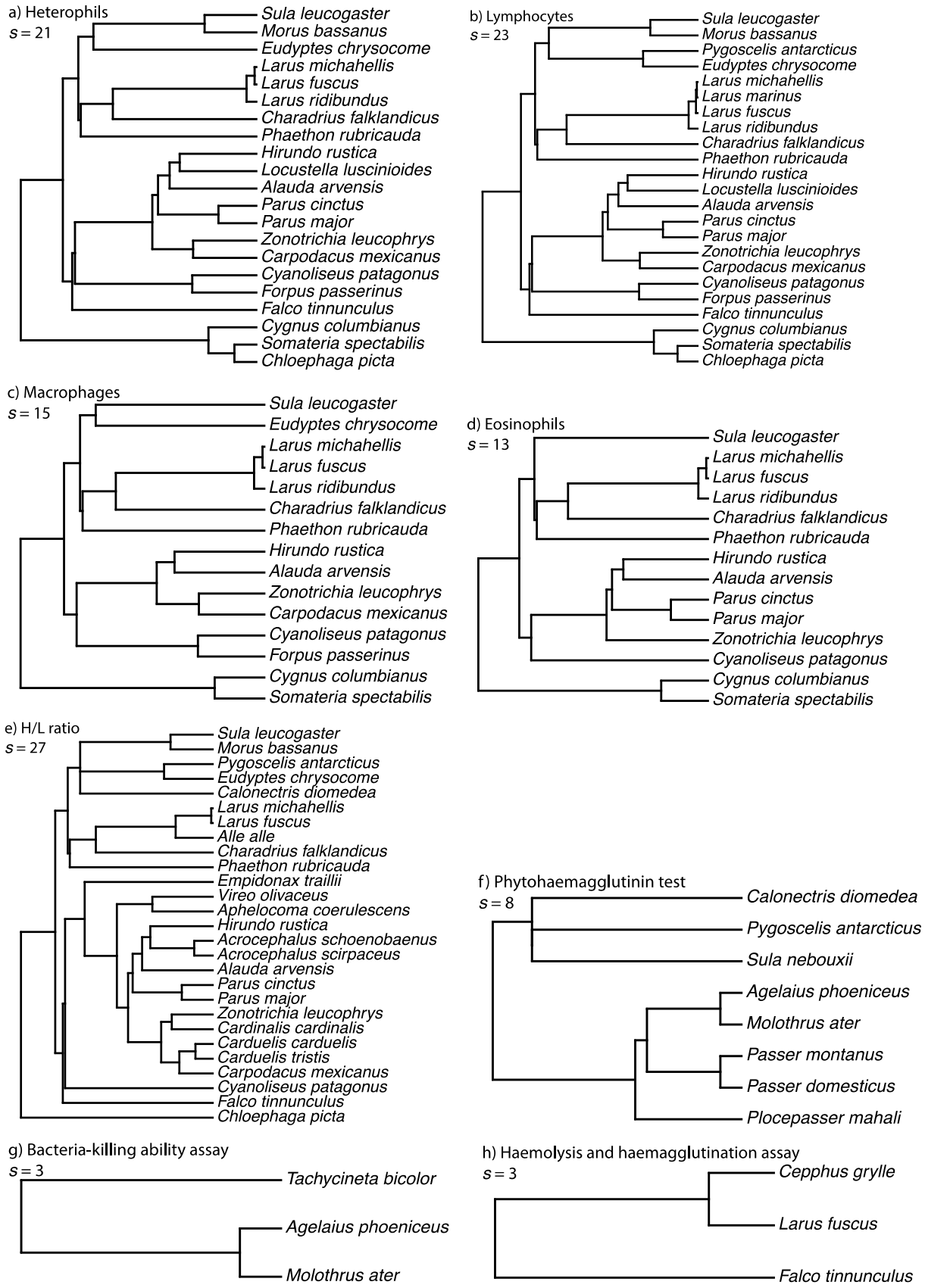

Figure S6.2 Phylogenetic trees used in meta-analyses and MCMC generalised linear mixed models. H/L ratio = heterophils/lymphocytes ratio. *s* refers to number of species.

Reference list of studies included in the meta-analysis

Cellular immunity (WBC)

1. Arriero et al. 2015. Variation in Immune Parameters and Disease Prevalence among Lesser Black-Backed Gulls (*Larus fuscus* sp.) with Different Migratory Strategies. PloS One 10: e0118279
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[1]The authors provided a dataset that was the source of the two publications cited.

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Immune response tests and assays

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Table S6.1 Breeding status and time from capture to blood sampling in studies investigating cellular immunity (WBC).

Reference number	Species	Breeding status	Breeding status	Stated capture-sampling time
1	*Larus fuscus*	Breeding	Captured at nest during breeding season	Not specified
2	*Zonotrichia leucophrys*	Breeding	Captured pairs with mist nets near the nest during breeding season. Breeding status confirmed with behavioural observations	Within 3 mins after capture
3	*Aphelocoma coerulescens*	Breeding	Not specified; sampled during the breeding season. Inferred by capture dateΨ	Within 1 min after capture
4	*Morus bassanus*	Breeding	Captured at nest during breeding season	Immediately after capture
5	*Hirundo rustica*	Breeding	Captured at the gate of breeding colony (in stables)	Not specified
6	*Haemorhous mexicanus*	Breeding	Not specified; sampled during the breeding season. Inferred by capture dateΨ	Immediately after capture
7	*Charadrius falklandicus*	Non-breeding	Captured with cannon net to non-breeding birds outside the breeding season	10–232 min, mean = 105.2 (SD = 56.7)
8	*Phaethon rubricauda* *Sula leucogaster*	Breeding	Captured at nest during incubation and chick rearing	Within 3–10 mins after capture
9	*Eudyptes chrysocome chrysocome*	Breeding	Captured during incubation	Within 3 mins after capture
10	*Larus michahellis*	Breeding Non-breeding	Captured at nest for breeders Captured outside the breeding season for non-breeders	Not specified
11	*Calonectris diomedea*	Breeding Non-breeding	Captured at breeding colony Divided in breeders and sabbaticals (non-breeders)	Not specified
12	*Chloephaga picta leucoptera*	Breeding	Captured during chick rearing with whoosh nets	Not specified
13	*Vireo olivaceus*	Breeding	Captured by mist-netting during the breeding season	Within 30 mins after capture
14	*Parus major*	Breeding	Captured from nest boxes during the breeding season	Not specified; stated: “at capture”

15	*Alauda arvensis*	Breeding Non-breeding	Captured breeding birds 2006–2009 captured birds at moulting, autumn migration and wintering 2007–2008	Within 2–35 min, median = 5 min
16	*Acrocephalus scirpaceus* *Acrocephalus schoenobaenus*	Breeding Non-breeding	Captured at different times of the year. The last one corresponding to post-breeding, dispersal and start of autumn migration	Not specified
17	*Spinus tristis*	Breeding	Mist nets by feeders in breeding grounds. Birds were determined as breeders based on bill and plumage colours	Within 60 mins after capture
18	*Parus major*	Breeding	Captured at nest boxes during breeding	Not specified; stated: “at capture”
19	*Poecile cinctus*	Breeding	Captured at nest boxes during breeding	Not specified
20	*Parus major*	Non-breeding	Captured with mist nets by feeders in winter	Within 1 min after capture
21	*Larus marinus*	Breeding	Captured at nest in breeding colony	Not specified; handling time and lymphocyte levels not correlated *P*-value = 0.34
22	*Locustella luscinoides*	Non-breeding	Captured with mist nest post breeding and beginning of dispersal and migration	Not specified
23	*Carduelis carduelis*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
24	*Cardinalis cardinalis*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
25	*Cygnus columbianus*	Non-breeding	Captured during moulting in the breeding grounds, previous start of migration	Not specified
26	*Pygoscelis antarcticus*	Breeding	Captured at nest in breeding colony	Not specified
27	*Larus ridibundus*	Non-breeding	Captured in non-breeding grounds (Spain)	Not specified
28	*Empidonax traillii extimus*	Breeding	Captured during breeding season. Presence of brood patch or cloacal protuberance	Not specified
29	*Pygoscelis antarcticus*	Breeding Non-breeding	Captured by hand at the nest during nesting for breeders and later at moulting for non-breeders	Not specified
30	*Hirundo rustica*	Breeding	Captured using mist nets when arriving to breeding site and then during check rearing	Not specified
31	*Falco tinnunculus*	Breeding	Captured at nest boxes during breeding season	Not specified; stated: “at capture”
32	*Cyanoliseus patagonus*	Breeding	Captured at nests on cliffs during breeding season	Within 30 mins after capture

33	*Somateria spectabilis*	Breeding	Captured at the onset of nesting during breeding season	Not specified
34	*Forpus passerinus*	Breeding	Captured at nest boxes or using mist nets close to nests	Not specified
35	*Alle alle*	Breeding	Captured at nest in the colony (stated that only took breeding adults)	Within 3 mins after capture

Ψ Species breeding period was assumed according to del Hoyo et al. (2019) Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona.

Table S6.2 Breeding status in studies investigating immune function.

Reference number	Species	Breeding status	Breeding status
1	*Larus fuscus*	Breeding	Captured at nest during breeding season
2	*Cepphus grylle*	Breeding	Captured when leaving nest in burrows in breeding colonies
3	*Plocepasser mahali*	Non-breeding	Captured at nest during breeding season; breeders were discarded after behavioural observations
4	*Calonectris diomedea*	Breeding	Captured by hand on their nests during the incubation, in colony
5	*Tachycineta bicolor*	Breeding	Captured from nest boxes during breeding season
6	*Passer montanus*	Breeding	Captured with mist nets and breeding status determined by anatomy and behaviour
7	*Molothrus ater* *Agelaius phoeniceus*	Breeding Non-breeding	Captured during the breeding season; only using older birds (according to plumage) to ensure they got a mate Recaptured outside the breeding season
8	*Passer domesticus*	Non-breeding	Captured just before start of breeding season. Reproductive status inferred by date of capture Ψ
9	*Pygoscelis antarcticus*	Breeding Non-breeding	Captured by hand at the nest during nesting for breeders and later at moulting for non-breeders
10	*Falco tinnunculus*	Breeding	Captured at nest boxes during breeding season
11	*Sula nebouxii*	Breeding	Captured at nest in breeding colony

12	*Agelaius phoeniceus*	Breeding	Captured during breeding season while territory formation till fledging of chicks

Ψ Species breeding period was assumed according to del Hoyo et al. (2019) Handbook of the Birds of the World Alive. Lynx Edicions, Barcelona.

Table S6.3 Number of studies, species and effect sizes used in present analyses.

Immune variable	Studies	Species	Effect sizes
White blood cells			
Heterophils	23	21	45
Lymphocytes	25	23	47
Macrophages	14	15	27
Eosinophils	12	13	24
H/L ratio	27	27	55
Immune response			
PHA	8	8	16
BKA	3	3	7
Haemolysis	3	3	3
Haemagglutination	3	3	3

H/L ratio = heterophils/lymphocytes ratio, PHA = phytohaemagglutinin test, BKA = bacteria-killing ability assay.

Model specification of Markov chain Monte Carlo simulations for generalised linear mixed models

Apart from the H/L ratio and the PHA, all our variables were proportions. Proportions were considered as success/failure (cbind(success, failure)) and the H/L ratio was log-transformed. The H/L ratio and PHA models were run with a Gaussian family distribution, whereas the rest of the models were run using a binomial family distribution (specified as 'multinomial2'). We used parameter expanded priors for the random effects (V = diag(1), nu = 1, alpha.mu = c(0), alpha.V = diag(1)), inverse Wishart priors (V = 1, n = 0.002) for the residuals and normal distributions centred on zero with large variances as fixed effects priors (default prior in function "MCMCglmm"). These priors were chosen to improve model convergence while being minimally informative (random effects) or completely uninformative (fixed effects). The models of macrophages in both full data set and subset analysis were run across 25,000,000 iterations with a thin of 20,000 and a burn-in of 5,000,000. The rest of the models were run across 1,603,000 iterations, thin of 1,600 and a burn-in of 3000. In all seven models, the potential scale reduction factor from the Gelman-Rubin test was 1.04 or lower, which is below the threshold of 1.1 indicating model convergence. Autocorrelation was also low, always below the threshold of 0.1.

Table S6.4 Number of species and number of individuals included in MCMC generalised linear mixed models of the full data set, and in the subset of data including species for which there were data from both the non-breeding and breeding periods.

	Analysis using full data set		Analysis using subset of data	
	Number of species	Number of individuals	Number of species	Number of individuals

Heterophils	21	90	3	28
Lymphocytes	23	94	4	30
Macrophages	15	56	2	14
Eosinophils	13	56	3	18
H/L ratio	27	110	7	48
PHA	8	32	5	24
BKA	3	14	2	12

Table S6.5 Sex difference and seasonal variation in white blood cell counts and immune responses in wild bird species for which there were data from both the non-breeding and breeding periods (MCMC generalised linear mixed models; *n* = total number of individuals; *P*-values <0.05 highlighted in bold).

		95% credibility intervals		
	Post. mean	Lower	Upper	*P*-value
a) Heterophils (*n* = 28)				
Intercept	-1.008	-2.261	0.424	0.124
Season (breeding)[a]	0.030	-0.630	0.676	0.946
Sex (males)[b]	-0.649	-1.145	-0.242	**0.004**
Season (breeding)[a]*sex (males)[b]	0.572	-0.052	1.213	0.084
Random				
Study	0.550	<0.001	1.852	
Phylogeny	0.592	<0.001	2.395	
Residual	0.123	0.014	0.265	
b) Lymphocytes (*n* = 30)				
Intercept	-0.048	-1.717	1.330	0.980
Season (breeding)[a]	-0.005	-0.563	0.556	0.994
Sex (males)[b]	0.334	-0.021	0.755	0.088
Season (breeding)[a]*sex (males)[b]	-0.280	-0.834	0.283	0.352
Random				
Study	0.260	<0.001	0.983	
Phylogeny	1.186	<0.001	3.588	
Residual	0.119	0.029	0.231	
c) Macrophages (*n* = 14)				
Intercept	-2.679	-5.357	0.317	**0.050**
Season (breeding)[a]	-0.598	-1.538	0.223	0.142
Sex (males)[b]	0.025	-0.501	0.496	0.930
Season (breeding)[a]*sex (males)[b]	0.241	-0.943	1.325	0.674
Random				
Study	1.786	<0.001	7.264	
Phylogeny	2.287	<0.001	10.91	
Residual	0.035	<0.001	0.145	
c) Eosinophils (*n* = 18)				
Intercept	-2.933	-4.824	-1.205	**0.018**
Season (breeding)[a]	0.410	-0.277	1.076	0.224
Sex (males)[b]	0.265	-0.292	0.838	0.346

Season (breeding)[a]*sex (males)[b]	-0.382	-1.278	0.411	0.376
Random				
Study	0.850	<0.001	3.622	
Phylogeny	1.138	<0.001	4.594	
Residual	0.05768	<0.001	0.207	
c) H/L ratio (*n* = 48)				
Intercept	-0.553	-1.353	0.183	0.120
Season (breeding)[a]	0.066	-0.342	0.492	0.744
Sex (males)[b]	-0.389	-0.750	-0.015	**0.040**
Season (breeding)[a]*sex (males)[b]	0.556	0.091	1.070	**0.040**
Random				
Study	0.394	0.021	0.989	
Phylogeny	0.177	<0.001	0.709	
Residual	0.212	0.126	0.306	
d) PHA response (*n* = 24)				
Intercept	0.805	-0.281	1.984	0.104
Season (breeding)[a]	-0.094	-0.334	0.153	0.422
Sex (males)[b]	0.091	-0.076	0.273	0.282
Season (breeding)[a]*sex (males)[b]	-0.070	-0.397	0.214	0.654
Random				
Study	0.224	<0.001	1.021	
Phylogeny	0.531	<0.001	1.881	
Residual	0.030	0.013	0.054	
d) BKA assay (*n* = 12)				
Intercept	-1.711	-6.111	2.292	0.234
Season (breeding)[a]	0.364	-1.213	1.989	0.624
Sex (males)[b]	-0.215	-1.414	1.110	0.702
Season (breeding)[a]*sex (males)[b]	1.501	-0.450	3.736	0.144
Random				
Study	3.328	<0.001	14.14	
Phylogeny	3.707	<0.001	11.33	
Residual	0.671	0.067	1.62	

[a]Relative to the non-breeding period

[b]Relative to females

Chapter 7 | Discussion

Sex roles, i.e. behavioural differences between males and females in mating and parenting, are believed to be the drivers of many sex differences in mortality in nature. Notable research in mammals has consistently shown that males die at higher rates and have shorter lifespans than females (Moore & Wilson, 2002; Lemaître et al., 2020). Interestingly, birds present the opposite pattern, with females in most species generally dying at higher rates than males do (Donald, 2007; Székely et al., 2014). The proximate causes of this female-biased mortality are not well understood and have reminded rather unexplained, perhaps because mortality is multifactorial, and the variables known to be related with mortality were first proposed in studies of taxa other than birds (e.g. testosterone immunosuppression or striking sexual size dimorphism, Moore & Wilson, 2002; Klein & Roberts, 2010). The main aim of my dissertation was to explore the relevance of parasite infection and immune variables in sex-specific mortality using birds as model organism.

My dissertation represents a contribution towards possible drivers of sex-specific mortality in vertebrates, going beyond previous studies in birds in two respects:

(iii) Unlike most studies available, I used two major predictors of adult mortality: parasite infection and the ability of immune system to respond to challenges.
(iv) I combined detailed species-specific studies with broader phylogenetic analysis across many bird species. This twofold approach enables, first, improve the definition of the associations in the species investigated (shorebirds), whiles the latter allows understanding the bigger picture.

Promislow et al. (1992) was the first study investigating sex-specific mortality across bird species, finding that females die at higher rates than males. This study was followed by Liker & Székely (2005) and Székely et al. (2014), who, using much larger data sets and improved methodologies, confirmed a female-biased mortality in birds. In this dissertation I expand on the knowledge of proximal variables that relate to sex-specific mortality in birds. My approach consisted on using variables known to directly or indirectly influence mortality and study whether they presented sex differences. This approach aimed to centre on pathogen and immune related variables (Smith et al., 2009; Froy et al., 2019), but not emphasising on life-history variables such as body size or adult sex ratio, already known to strongly correlate with sex-specific mortality, but far from explaining the true causes of sex-specific mortality (Promislow et al., 1992; Moore & Wilson, 2002; Liker & Székely, 2005; Székely et al., 2014).

7.1 The role of parasites in sex-specific mortality

Mortality events are difficult to accurately determine in the wild because they are often the result of several processes. For example, parasite infection has been associated with reduced survival in gastropods, frogs, and

birds (Cunningham & Daszak, 1998; Johnson et al., 1999; Lachish et al., 2011), although parasites are seldom the exclusively cause of mortality. In fact, the very definition of parasite describes organisms that live at expenses of the host, causing some harm, but not killing it. Exceptions are cases of extreme parasite load, usually in immunosuppressed individuals, or in events of parasites infecting naïve hosts (commonly seen on islands, e.g. Kleindorfer et al., 2014). Certain bacteria stains are known for being pathogenic when colonising a new host, but bacteria adaptation to evade immune recognition and thus avoid host repulsion also occurs (Atterby et al., 2018; Chaguza, 2020). Adaptation could explain the results observed in Chapter 2 as prevalence of bacteria infection did not predict body condition. In Chapter 2 I also found sex differences in prevalence in Kentish plover: females had higher bacteria prevalence than males. This sex bias, interestingly, was in accordance with the female bias in mortality that most populations of Kentish plover show (Eberhart-Phillips et al., 2018). Whether different bacteria infection between the sexes is associated to sex-specific mortality or not is for future studies to unveil, although the negative impact of infection disease in wildlife is widely recognised (Delahay et al., 2009; Smith et al., 2009; Heard et al., 2013).

Sex-specific burden of macro-parasites and the impact of parasite on host survival are well-described in animals (Poulin, 1996; McCurdy et al., 1998; Moore & Wilson, 2002; Karvonen & Lindström, 2018). Therefore, it is notable that in Chapter 3, parasite prevalence across 138 bird species was unrelated to annual mortality in males, females, and the sex bias. Body size positively correlates with parasite burden, thus the results in Moore & Wilson (2002) could partly be explained by the more striking sexual size dimorphism (SSD) in mammals compared to birds (Fairbairn et al., 2007). However, perhaps a limitation using prevalence as variable could have also obscured my findings, as this variable could be subject of a process known as survivorship bias which, in this context, corresponds to the logical error of determining parasite prevalence from individuals that have already survived the infection (Zens & Peart, 2003).

Now, if we assume that the potential problems mentioned above are minor, an alternative interesting approach that could be adopted to further understand the relationship between parasitism and bird survival derives from reflecting upon the predator-prey interaction. If we consider that indirect causes of mortality by parasites are major (Toscano et al., 2014; Adelman et al., 2017), it would be expected that bird species with high risk of predation should perceive a higher impact of parasite infection because their survival dramatically depends on their fleeing ability, one of the main anti-predator strategies in birds (Barnard, 1983; Poulin & Maure, 2015; Adelman et al., 2017). However, this idea needs thinking because is not trivial to identify a consistent proxy of predation risk across species. One could argue that perhaps non-carnivores are the usual subject of predation, but carnivores are can also suffer intense predation. Perhaps body size could work, as Hill et al. (2019) found a relationship between the risk of predation and body size, showing that small-bodied birds had higher predation rates than large-bodied ones. In any case, I think this predator-prey interaction is worth considering for expanding the understanding how parasites and mortality interact in wild birds.

7.2 Immune defence and sex-specific mortality

Immune function is a complex process and its intricate mechanisms are energetically costly (Sheldon & Verhulst, 1996; Hasselquist & Nilsson, 2012). Moreover, many of the costs associated with disease, for instance fever, inflammation and septic shock, are not direct costs of the infectious organism but rather the result of the activation of the immune defence (Janeway et al., 1999; Levin & Antia, 2001; Marais et al., 2011; Cabrera-Martínez et al., 2018). Energy is a limited resource in wild animals and often proposed as currency in trade-off/investment hypotheses linking the immune system and other costly life-history traits (Lochmiller & Deerenberg, 2000; Bonneaud et al., 2003; Lee et al., 2006; Nilsson et al., 2007). From this perspective, there are many views that support sex differences in immunity, and moreover, would explain observations of sex-specific mortality in birds. For example, in Kentish plovers, most of their populations present a balanced hatching sex ratio, although have more males than females in the adult pool since females tend to die at higher rates than males (Székely & Lessells, 1993; Fraga & Amat, 1996; Székely & Cuthill, 1999; Székely et al., 2004; Maher et al., 2017; Eberhart-Phillips et al., 2018; Que et al., 2019). These consistent life-history and demographic parameters would suggest females as the sex likely to present weaker immune defences in this shorebird. However, the results in Chapter 4 did not support the latter expectation, finding no sex differences in haemolysis and haemagglutination assays in two shorebird species. Note that the specific hypothesis of Chapter 4 was better suited for immune tests evaluating the immune response rather than immune function (i.e. magnitude of an immune response to a challenge *versus* baseline immunity, respectively). I also note that the immune system has great diversity, with several parameters that could be measured, but this would not be cost efficient. To characterise immune system, methods like gene expression might be advantageous because they offer a much thorough view of biological processes such as immune responses. Accordingly, in Chapter 5 I found that expression of immune genes showed a small but significant male bias. Perhaps the true effect of sex on immune defence is small, so that data from more individuals and species –and better controlled assays– would be needed.

The breeding period has strong repercussions on many aspects of the bird's physiology. Chapter 6 shows that the immune system is not exception to this as it seems that only during breeding, males tend to show stronger immunity than females. If a better immune system improves survival (Hegemann et al., 2013; Froy et al., 2019), then our results match the across species female-biased mortality pattern in birds. Future studies should investigate whether these findings are more than just a coincidence from a multi-species perspective. Chapter 6 could bring further repercussion in sex-specific mortality in wild animals because the fact that sex-specific strength of immune defence depended on breeding status suggests an additional layer of complexity in matters of survival cost of immune defence. One could argue that the impact of immune function during the non-breeding season could be even more important because in most birds the breeding season is rather short compared to the non-breeding season. Future studies should address the impact on survival of immune defence during the non-breeding period, as well as evaluating if it differs to that of the breeding period.

7.3 Bird mortality in the wild

Naturally, mortality is not limited to the variables my dissertation has evaluated. A meta-analysis of 6860 individual mortalities of adult free-living birds (excluding direct anthropogenic causes) showed that the main causes of death were predation (68%), disease (3%), collision (1%), starvation (1%), and other (4%, Hill et al. 2019). While another study examining 1,005 bird carcasses in the wild showed that the main causes of death were physical injury (32.6%), systemic disease (13.5%), parasitic disease (11.6%), poisons (9.6%), bacterial disease (7.8%), viral infections (6.2%), fungal infections (1.8%), and 17% deaths from unknown causes (Jennings, 1961). More recent studies highlight collisions with objects, and predation by domestic cats as other important sources of mortality in birds, particularly in urbanised environments (Erickson et al., 2005; Loss et al., 2015). From these data we could confirm that the variables evaluated in this dissertation are an important part of mortality in wild birds, as the immune system, parasites and bacteria can be linked to a great fraction of deaths (i.e. predation, physical injury, systemic, parasitic, and bacterial disease, viral and fungal infections).

In my opinion, the most important variable that I left untouched in the present dissertation was predation. However, can be argued that the mortality cost of parasites and the immune system could be importantly determined by changes in behaviour of the host, reducing their anti-predator response. A recent study provided a good example of these indirect means of mortality associated to pathogen-host dynamics. Adelman et al. (2017) showed that House finches (*Haemorhous mexicanus*) infected with *Mycoplasma gallisepticum* –cause of conjunctivitis and reduced survival among free-living birds– experienced reduced anti-predator response compared to the control group, thus providing a plausible explanation as of why this bacteria rarely causes mortality in House finches in captivity. While the above example does not include sex differences mortality, is not difficult to imagine the sex-specific impact if susceptibility to infection would show sex differences. Additionally, sex-specific predation could be driven by different colouration, size, and behaviour between males and females, where males are usually more conspicuous and larger than females.

Sex differences in mortality is certainly a topic difficult to disentangle given the wide range of relative processes that could influence it. Overall, this dissertation represents a relevant contribution to this topic, in the specific case of birds. As is often expected in research, many limitations arose during the confection of this research work, but nevertheless it was possible to generate sensible conclusions about the matter and more importantly, stablish possible directions in future research.

7.4 Future directions

In this dissertation I explored the potential impact of parasite burden and aspects of the immune system, in association with several life-history variables, on sex differences in mortality in wild birds. This is initial exploration allowed me to envision a number of research topics that have not yet been investigated.

1. The first logical future venture is to expand the work presented in Chapter 4. Despite the specific limitation of shorebird fieldwork, immune assays that require plasma preservation seem promising. In order to further understand the sex-specific trade-offs associated with investing in attraction *versus*

investing in longevity/survival. Sampling both chicks and adults would extend the validity of my results. Also, I would aim to widen the species range of shorebirds to cover more mating strategies (polyandrous, monogamous, polygynous). Many shorebirds have their mating strategies and demographic parameters well-studied, thus enabling formulating specific predictions of the direction of the immune defence biases.

2. The impact on survival associated to interspecific variation in immune strength in birds is well documented (Møller & Saino, 2004; Hegemann et al., 2013; Tieleman, 2018). Another interesting approach consists on determining the sex-specific influence of immune defence on survival of Kentish plover. Ideally, this research will include a well-studied system such as the Kentish plover that exhibit different adult sex ratios and sex-specific mortalities in order to understand the drivers of adult sex ratio variation.
3. The immune system in male and female birds appears to vary with season (Chapter 6). The impact of immune defence on survival is well known but most of studies report results conducted in birds sampled during the breeding season. It would be interest to know if the impact on survival differs between the specific immune status during the breeding *versus* non-breeding seasons. One could argue that the impact of immune defence on survival during breeding could be minor compared to the non-breeding period that is much longer. However, I recognise that studying this topic could be difficult because there are numerous variables that could confound the associations, such as food availability and inclement weather.
4. Bio-medical research is moving fast toward genomic approaches. Recent experimental research has provided important contribution in the understanding of host response to pathogen infection. Notably, Videvall et al. (2015) and Scalf et al. (2019) showed using transcriptomic analysis the immune response to blood parasite infection in Eurasian siskins (*Carduelis spinus*), and to bacterial lipopolysaccharide in Zebra finch (*Taeniopygia guttata*). Note that none of these studies focussed on sex differences, and therefore, there is a niche to move in. I believe that there is great potential in exploring sex- and age-specific differences considering that recent studies show an important role of sex-specific chick mortality in predicting later adult sex ratio (Eberhart-Phillips et al., 2018). Furthermore, these transcriptomic tools will allow exploring alternative aspects of the birds' biology that could be related to mortality such as stress susceptibility that, according to the work of Wang et al. (2019) in captive Eurasian magpies, also present sex biases in gene expression, being higher in females than in males.
5. Finally, one could also conjecture about broad-scale associations between parental care, survival and immune system that may emerge how critical the survival of a parent is (either the male or the female), for the survival of the young.

   For example, in placental mammals, conception and embryonic development takes place in the females' womb. This simple fact determines females as more important for offspring survival compared to males, whose only (but not exclusive) role often is to provide the sperm. This hypothesis could be seen as a generalisation of the Pregnancy Compensation Hypothesis presented for humans (Natri et al., 2019). Since the offspring depends completely on the care of females during initial stages of its life, for food (lactation, nutrition and immune defence from colostrum) and for protection. Therefore, it can be assumed that

natural selection possibly favoured the development of a stronger and efficient immune system in females but not in males, because is crucial that females survive pregnancy and lactation, whiles often males need to live long enough to mate.

Accordingly, male and female mammals are expected to show strong differences in immunocompetence, lifespan and disease susceptibility, where females are widely recognised as the sex with the strongest immune defence, lower rates of mortality as well as longer lifespan (Klein & Roberts, 2010; Klein & Flanagan, 2016; Lemaître et al., 2020).

In birds and other egg-laying tetrapods, the relationships may differ since the fertilisation of the eggs may occur within the reproductive tract of the female although offspring development takes place outside the mother. For example, in birds, soon after copulation and fertilisation has occurred, the female will lay the eggs. From this point on, the survival of the young will depend importantly on the parental care duties. Parental care varies greatly among species, but most bird present biparental or female-biased parental care although male-biased care also occur in certain taxa such as ratites, coucals and shorebirds (Bennett & Owens, 2002).

I conjecture that sex biases in survival-related immune variables in tetrapods should be associated with the degree of sex-specific parental investment, used as proxy of the relevance of each sex for survival their offspring(s). Because the reproductive success depends upon survival of the young, selection will favour traits that allow higher survival of the sex that provides most of the parental care, i.e. strong immune defence (health), cryptic coat (predation). In species where one sex is prominently more indispensable than the other, like female mammals, these sex differences in immunity are expected to be more noticeable. Whereas in other taxa like birds, where the parental duties are more shared, sex differences in immune system are expected to be weaker.

In conclusion, the five chapters in my dissertation attest the importance of understanding sex roles, sex-different demographies and immune system using a coherent framework. Whilst pursuing the objectives of my dissertation, I have gained critical knowledge on the relevance of pathogen-host dynamic, and how it could affect sex differences in mortality. To understand the dynamics of big patterns in nature is necessary to implement phylogenetic comparative analysis otherwise is easy to fall in wrong assumptions based on extrapolations of other taxa. Sex-specific research has gained great relevance during the recent years; is critical to continue such investigation in order to obtain better resolution in the events of nature.

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Appendix 1 | Mate fidelity in a polygamous shorebird, the snowy plover (*Charadrius nivosus*)

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Mate fidelity in a polygamous shorebird, the snowy plover (*Charadrius nivosus*)

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Abstract

Social monogamy has evolved multiple times and is particularly common in birds. However, it is not well understood why some species live in long-lasting monogamous partnerships while others change mates between breeding attempts. Here, we investigate mate fidelity in a sequential polygamous shorebird, the snowy plover (*Charadrius nivosus*), a species in which both males and females may have several breeding attempts within a breeding season with the same or different mates. Using 6 years of data from a well-monitored population in Bahía de Ceuta, Mexico, we investigated predictors and fitness implications of mate fidelity both within and between years. We show that in order to maximize reproductive success within a season, individuals divorce after successful nesting and re-mate with the same partner after nest failure. Therefore, divorced plovers, counterintuitively, achieve higher reproductive success than individuals that retain their mate. We also show that different mating decisions between sexes predict different breeding dispersal patterns. Taken together, our findings imply that divorce is an adaptive strategy to improve reproductive success in a stochastic environment. Understanding mate fidelity is important for the evolution of monogamy and polygamy, and these mating behaviors have implications for reproductive success and population productivity.

KEYWORDS
breeding dispersal, *Charadrius nivosus*, divorce, mate fidelity, nesting success, polygamous

Krisztina Kupán and Tamás Székely are co-authors shared senior authorship to this work.

Appendix 2 | Successful breeding predicts divorce in plovers

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Successful breeding predicts divorce in plovers

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When individuals breed more than once, parents are faced with the choice of whether to re-mate with their old partner or divorce and select a new mate. Evolutionary theory predicts that, following successful reproduction with a given partner, that partner should be retained for future reproduction. However, recent work in a polygamous bird, has instead indicated that successful parents divorced more often than failed breeders (Halimubieke et al. in Ecol Evol 9:10734–10745, 2019), because one parent can benefit by mating with a new partner and reproducing shortly after divorce. Here we investigate whether successful breeding predicts divorce using data from 14 well-monitored populations of plovers (*Charadrius* spp.). We show that successful nesting leads to divorce, whereas nest failure leads to retention of the mate for follow-up breeding. Plovers that divorced their partners and simultaneously deserted their broods produced more offspring within a season than parents that retained their mate. Our work provides a counterpoint to theoretical expectations that divorce is triggered by low reproductive success, and supports adaptive explanations of divorce as a strategy to improve individual reproductive success. In addition, we show that temperature may modulate these costs and benefits, and contribute to dynamic variation in patterns of divorce across plover breeding systems.

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Appendix 3 | Breeding ecology of the Cream-coloured Courser in Cape Verde

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Breeding ecology of the Cream-coloured Courser in Cape Verde

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The Cream-coloured Courser *Cursorius cursor exsul* is a data-deficient shorebird distributed across Eurasia and Africa. The subspecies *exsul* is endemic to the Cape Verde archipelago. In contrast with their mainland breeding sites, where coursers appear to be nomadic and rare throughout their range, the Cape Verde population is year-round resident and locally abundant. Here we investigate the breeding ecology of Cream-coloured Coursers in Maio, Cape Verde, where they breed in rocky semi-desert habitat. Over four consecutive breeding seasons (2015–2018), we found 52 nests, and ringed 56 adults and 100 chicks. Forty eight percent of 52 nests produced at least one chick; the main nest predators were Brown-necked Ravens *Corvus ruficollis* and domestic dogs *Canis familiaris*. Although coursers were thought to be sexually monomorphic, we found that adult males had longer tarsi than adult females. Coursers appeared to be socially monogamous and both sexes incubated the eggs and reared the young. Maio is currently a rural island with little development; however, the island is faced with the threat of touristic development. Therefore, research is required to understand how the courser population will respond to anthropogenic pressures in the future.

Écologie de reproduction de la Courvite isabelle au Cap Vert

La courvite isabelle *Cursorius cursor exsul* est oiseau de rivage distribué en Eurasie et en Afrique et pauvre en données. La sous-espèce *exsulis* est endémique de l'archipel du Cap-Vert. Contrairement aux sites de reproduction continentaux où les courvites semblent être nomades et rares dans l'ensemble de leur aire de répartition, la population cap-verdienne est résidente toute l'année et est localement abondante. Nous étudions ici l'écologie de la reproduction des courvites à Maio, au Cap-Vert, où ils se reproduisent dans un habitat rocheux semi-désertique. Au cours de quatre saisons de reproduction consécutives (2015–2018), nous avons trouvé 52 nids et bagué 56 adultes et 100 poussins. Quarante-huit pour cent des 52 nids ont produit au moins un poussin. Les principaux prédateurs de nidification étaient le corbeau brun *Corvus ruficollis* et les chiens domestiques *Canis familiaris*. Bien que les courvites aient été considérés comme sexuellement monomorphes, nous avons constaté que les mâles adultes avaient un tarse plus long que les femelles adultes. Les courvites semblaient être socialement monogames et les deux sexes couvaient les œufs et élevaient les jeunes. Maio est actuellement une île rurale avec peu de développement, cependant, l'île est menacée par le développement touristique. Par conséquent, des recherches sont nécessaires pour comprendre comment cette population de courvite isabelle répondra aux pressions anthropiques à l'avenir.

Keywords: breeding success, *Cursorius cursor exsul*, mating system, parental care, sexual size dimorphism

Introduction

Desert regions represent up to one third of the land surface on Earth. Despite its extent, little biodiversity can subsist, as a result of its extreme environmental conditions (Ward 2009). Accordingly, desert dwelling species usually present physiological adaptations that enable them to survive under intense solar radiation, low relative humidity and meagre primary productivity. Desert birds, for instance, show a reduced water loss, reduced metabolic rate, and small clutch size (Williams and Tieleman 2005). Climate change, expansion and intensification of arable agriculture, mining deserts for fuels, and habitat loss or fragmentation are some of the factors threatening desert dwelling species (Ayyad 2003; Kamp et al. 2016).

The Cream-coloured Courser *Cursorius cursor* is a ground-nesting desert wader from the Family Glareolidae. Based on morphological differences; three subspecies are currently recognised. The nominate race *C. c. cursor* is distributed across arid regions of Northern Africa, the Arabian Peninsula and the Canary Islands, *C. c. bogolubovi* is found in the Middle East, and *C. c. exsul* is restricted to the Cape Verde archipelago (Tavares 2013; Maclean and Kirwan 2019). Despite the wide distribution and breeding

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Appendix 4 | Report on beak abnormalities of some birds of Patagonia

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Report on beak abnormalities of some birds of Patagonia

José O. Valdebenito,[1]* Alexandra Grandón-Ojeda,[2] Vicente Pantoja-Maggi,[3] Fernando J. Novoa,[4] and Daniel González-Acuña[2]

ABSTRACT—Avian beaks are complex and highly specialized structures that if altered could hinder many aspects of bird biology. Here, we provide evidence from incidental sightings of 24 birds from 9 species presenting a mild to severe degree of beak abnormality, including species from Passeriformes, Falconiformes, Sphenisciformes, and Charadriiformes, recorded in Chile (2013–2016). The most common alterations corresponded to crossed beaks and excessive elongation of the upper beak (25% each). The Austral Thrush (*Turdus falcklandii*; $n = 11$) and Chilean Mockingbird (*Mimus thenca*; $n = 5$) were the birds most frequently recorded with abnormalities. With the exception of the Austral Thrush and Magellanic Penguin (*Spheniscus magellanicus*), all beak abnormalities mentioned here are the first recorded for each species. *Received 11 August 2017. Accepted 18 September 2018.*

Key words: bill, collisions, crossed-beak, deformity, South America.

Reporte de anormalidades de picos en algunas aves de la Patagonia

RESUMEN (Spanish)—Los picos de aves son estructuras complejas y altamente especializadas que, si se ven alteradas, pueden perjudicar muchos aspectos de la biología del ave. En este trabajo proveemos evidencia basada en observaciones incidentales de 24 aves correspondientes a 9 especies presentando deformidades de pico desde leves a severas, incluyendo individuos de las familias Passeriformes, Falconiformes, Sphenisciformes, y Charadriiformes, registrados en Chile (2013–2016). Las alteraciones más comunes correspondieron a picos cruzados y elongación excesiva de la maxila superior (25% cada una). El Zorzal Patagón (*Turdus falcklandii*; $n = 11$) y el Sinsonte Tenca (*Mimus thenca*; $n = 5$) fueron las aves con mayor frecuencia registradas con anormalidades. Con la excepción del Zorzal Patagón y el Pingüino Magallánico (*Spheniscus magellanicus*), todas las anormalidades aquí mencionadas corresponden a primeros registros para cada especie.

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Palabras clave: América del Sur, colisiones, deformidad, pico, pico cruzado.

In addition to its evident role in food intake as the beginning of the gastrointestinal system, the beak is essential in many other aspects, including feather maintenance and parasite control (Clayton et al. 2005), social behavior (Zampiga et al. 2004), and navigation (Wiltschko and Wiltschko 2013). Abnormalities in beak shapes are often the result of pathologies affecting the beak structure. Many factors have been associated with these deformities in domestic birds, for example dietary deficiencies of vitamins or calcium, specific gene mutations, viruses, and abnormal feeding of the nestling (Gylstorff and Grimm 1987, Palya et al. 2009, Bai et al. 2014). These causes, however, not always can be applied to wild birds, where injury to the beak, parasitic or pathogenic infection, pollution, and toxins are often hypothesized as etiologic agents of, for instance, abnormal growth of the outer keratin layer and congenital malformations (Arendt and Arendt 1986, Bowerman et al. 1994, Galligan and Kleindorfer 2009). The most well-documented recent event was reported by Handel et al. (2010) affecting hundreds of birds in Alaska and elsewhere in North America. The etiology of this epizootic event seemed to be linked to a novel Picornavirus and a Poecivirus, both isolated from a population of affected Black-capped Chickadees (*Poecile atricapillus*; Zylberberg et al. 2016, 2018).

Despite the rich avian biodiversity of South America and the importance that beak abnormalities could have on avian life history, few records of beak abnormalities have been published. Here, we provide a detailed description of 9 species belonging to 4 avian orders recorded in Chile presenting different degrees of beak abnormalities.

Appendix 5 | Gastro-intestinal microbiota of two high altitude bird populations

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Manuscript in preparation

Abstract

Gut microbiotas contribute to shaping reproductive success, health and lifespan. Extensively studied in humans and lab animals, gut microbiotas have been increasingly explored in a wide range of taxa in the wild. Environment, sex and age influence the gut microbiota but whether this is due to intrinsic processes or knock on effects from behaviour remains unclear. Here, we provide an insight into the composition of gut microbiota in Kentish plover (*Charadrius alexandrinus*) and lesser sand plover (*Charadrius mongolus*) in natural populations. By analysing faecal bacterial communities using 16S rRNA high-throughput sequencing technology, we find that both species have a similar and overlapping gut microbial communities with Firmicutes, Proteobacteria and Bacteroides being the dominant phyla, suggesting that similar breeding environment nay be a factor shaping gut microbiota. In Kentish plovers, we found no evidence of age- and sex-related differences. Our study provides baseline information that can be used in future studies to better understand diversity, function and determinants of gut microbes in *Charadrius* shorebirds.

Keywords: Gut microbiota, 16sRNA gene, shorebirds, environment, social structure, disease biology, mating system, development

1. Introduction

Microbiome research is an emerging field in ecology and evolutionary biology. The gut microbiota, is a diverse community of bacteria, archaea or eukaryote that resides within the gastrointestinal tract, influencing the physiology, behaviour, and fitness of the host (Ley et al. 2008, Kohl 2012, Waite and Taylor 2015). Since the gut microbiota may be pivotal to important biological processes, understanding factors that shape overall gut microbiota biodiversity is of utmost interest.

Several studies have looked at the factors that shape gut microbiota, host phylogeny, environment, sex and development have been identified as important (Foster et al. 2017, Grond et al. 2018, McDonald et al. 2018, Watson et al. 2019). Gut microbial communities are more similar among individuals of the same species than among individuals of different species (Ley et al. 2008, Goodrich et al. 2014, Waite and Taylor 2014, Hird et al. 2015). The environment however has a significant effect on gut microbiota composition. For instance, individuals from sympatric species of migratory passerines (Lewis et al. 2017) and non-human primates (i.e. chimpanzees and gorillas; Moeller et al. 2013) show convergent gut microbial communities reflecting their similar ecological environment and diet. In contrast, individuals from populations at different locations show differences in gut microbiota. For example, gut bacterial diversity is significantly higher in onshore polar bears compared to offshore polar bears (Watson et al. 2019). Migrants consistently had higher abundances of certain bacterial genus compared to conspecific residents in sandpipers (Risely et al. 2018).

Gut microbiota of males and females differ in several species including humans (Elderman et al. 2018, Kim et al. 2020). Gut microbiota sex differences can result from intrinsic physiologic sex differences but may also be indirectly caused by behavioural differences between the sexes. If the former, then it could be expected that patterns of differences between males and females should be consistent across species. If the latter, gut microbiota patterns in males and females should vary according to behavioural differences in species with different social behaviours. Most studies have suggested the sex differences in gut microbiota stem from sex-dependent physiological conditions (e.g. sex hormone and body mass index) and diet (Flores et al. 2012, Bolnick et al. 2014, Dominianni et al. 2015, Neuman et al. 2015, Org et al. 2016, Escallon et al. 2017). Another line of studies have shown that social contact can mediate the acquisition and flow of microbiomes between individuals (Degnan et al. 2012, Song et al. 2013, Nuriel-Ohayon et al. 2016), social contacts may allow for the horizontal gut microbiota transmission, which is presumed in direct relation to the patterns of sex-specific social contacts (e.g. social interactions, mating behaviours; Münger et al. 2018). For example, in wild black howler monkeys (*Alouata caraya*), gut microbiotas of adult females that generally interact more with each other showed greater similarity compared to males that have fewer social contact with both females and other males (Tung et al. 2015, Amato et al. 2017). In lizards, the cloacal bacterial communities (cloacal bacterial samples are widely used as a proxy of gut microbiota in studies of birds and reptiles; Videvall et al. 2018) of polyandrous female were significantly more diverse than in monogamous females, suggesting that a larger number of sexual partners increased bacterial diversity in females' cloaca (White et al. 2011). Other studies support these findings, reporting higher microbial diversity in the sex with the highest diversity of social or

ecological niches (White et al. 2010, White et al. 2011, Leclaire et al. 2014, Levin et al. 2016, Amato et al. 2017).

Several studies have reported changes in gut microbiota along development (Cox et al. 2012, Faith et al. 2013, Grond et al. 2017, Videvall et al. 2018). For example, gut microbial communities during early life in the young are highly variable in diversity and abundance and are markedly different from gut microbiotas of conspecific adults (González-Braojos et al. 2012, van Dongen et al. 2013, Waite and Taylor 2014). However, the mechanism behind age-related gut microbial difference is poorly studied, such differences can be caused by parenting behaviour (e.g. biparental care vs. uniparental care). In several species, there is evidence of vertical transmission from mother to offspring playing an important role in the establishment of gut microbiotas during early life offspring (Nuriel-Ohayon et al. 2016). The extent of vertical transmission is thought to be at least partly dependent on the precocity of the young. For example, in altricial birds where young are usually dependent on their parent(s) for food, gut microbiota might be inoculated through feeding, therefore, the offspring's gut microbiota should resemble the parent(s)'. Precocial young leave the nest soon after hatch and often forage independently, thus there would be fewer opportunities for parent to offspring gut microbiota transmission.

Charadrius plovers, are a group of small ground-nesting shorebirds breeding on all continents except Antarctica (Eberhart-Phillips 2019). Kentish plover (*Charadrius alexandrinus*) and lesser sand plover (*Charadrius mongolus*) breed at an alkaline lake in Tibetan Plateau (3200m above sea level) from May to July, and winter in coastal southern China. Both species are monogamous, males and females have roughly a similar number of close partners. They both show biparental care to the precocious offspring. They share common breeding habitat and forage mainly on aquatic insects and their larvae (Fieldwork observation). They represent a good system to test the influence of environmental and social behaviour in gut microbiota.

Here, we analyse microbial biodiversity in faecal samples to assess gut microbial community female, male and young individuals in wild populations of Kentish and lesser sand plover to assess relative relevance of genetic differences between species, social links or environment in shaping the gut microbiota in these two species. Here we explore three hypotheses that assume distinct major drivers of gut microbiota diversity and composition at different scales. First, if the gut microbiota composition is mainly determined by underlying genetics, we would expect significant differences in microbial communities between the two species analysed. However, if environment conditions are the main force to shaping gut microbiota composition, we expect gut microbiota composition to be similar in both species as both inhabit the same environment during their breeding season. Second, if physiological differences between the sexes influence gut microbiota, then we expect male and female microbiomes to be different. However, if sex differences are explained by differences in social networks, home ranges and foraging behaviour, then we expect that microbiomes in these monogamous and biparental caring birds to be similar for males and females. Third, if differences in gut microbiota between adults and juveniles are explained by intrinsic differences in the physiology of the two life stages, then we would expect to observe differences in the two species included in this study. However, if these differences are the result of the differences in diet of adults and young (i.e. in mammals, young are fed mother's

milk and in birds, many young are provided with food by parent(s)), we would expect that gut microbiota composition should not differ between adults and juveniles in these two precocial species where young fetch their food by themselves.

2. Materials and Methods

2.1 Sample collection

Fieldwork was carried out at Qinghai Lake, an alkaline lake lies in Tibetan Plateau, in May and June 2019. Kentish plovers and lesser sand plovers breed along the lake shore. Breeding pairs were captured on their nest while incubating eggs, using funnel traps (Székely et al. 2008). The sex of adult birds was determined by morphological features, and molecular sexing was applied to identify the sex of the chicks. Microbiota samples were collected from captured adults and chicks. Faecal samples are generally representative of the bacterial community in the large intestines. We adopted Knutie and Gotanda's (2018) faecal sampling protocol. The captured bird was put into a paper bag with a sterile wax paper on the bottom. A metal grate was set over the wax paper to prevent the bird from directly encountering the faeces after defecation. After defecation, faecal samples were collected using sterile polyester swabs, placed in sterile cryotubes without medium, and kept in liquid nitrogen. The grate was sterilized by soaking them in a 10% bleach solution for at least 10 min before each collection to reduce potential cross-contamination between different individuals. The bird was held for less than 5 min to minimize stress and then be released. Samples were transported from the liquid nitrogen to a laboratory freezer at -80 °C. As studies suggest differences in bacterial composition resulting from storage conditions do not eclipse differences between samples, even when left at ambient temperatures for 2 weeks (Lauber et al. 2010, Dominianni et al. 2014, Song et al. 2016), we assume that changing of the storage environment had minimal effect on microbial composition of the samples collected.

2.2 DNA isolation, amplification and sequencing

To obtain sufficient amounts of genomic DNA for sequencing library preparation, the fecal sample on the sterile tips were used to extract genomic DNA with the FastDNA Spin Kit for Feces (MP Biomedicals Co., Ltd., USA) following the manufacturer's instructions. Then, the concentration and purity of the extracted DNA samples were measured using a Nanodrop 2000 spectrometer (Thermo Fisher Scientific, Wilmington, DE, United States). Bacterial diversity was examined after 250-bp paired-end amplicon sequencing using the primers 515F (5- GTGYCAGCMGCCGCGGTAA-3) and 806R (5-GGACTACNVGGGTWTCTAAT-3) on an Illumina Miseq platform in two separate runs at Suzhou GENEWIZ, Co., Ltd, (Suzhou, China).

Paired-end reads were merged, and pairs diverging by less than 200 bp or containing unknown base calls (N) were discarded. Then the PCR amplification primers were trimmed, and the sequences were quality filtered at 0.5% Expected Error (EE); those displaying greater than 0.5% EE were discarded. Using the VSEARCH (ver. 1.9.6) algorithm, OTUs were created by clustering sequences with 97% sequence identity, discarding chimeric sequences after being aligned to the SILVA reference. Taxonomic assignments of

representative sequences from each OTU were performed using the Ribosomal Database Program (RDP) Bayesian algorithm. Sequences with ≥ 97% similarity were assigned to the same OTU.

2.3 Statistical analyses

All the faecal samples were classified into three groups: (a) species group (Kentish plover vs. lesser sand plover); (b) sex group (female Kentish plover vs. male Kentish plover); (c) age group (adult Kentish plover vs. juvenile Kentish plover). We analysed bacterial communities in three ways. (1) To identify the OUTs that differ in abundance within each comparison group, we applied the Metastat gap analysis. (2) To examine community-wide differences in abundance within each comparison group, we fitted non-multidimensional scaling (NMDS, vegan package in R; R Core Team 2018) ordinations to rarefied count data, the results were presented based on Bray-Curtis (based on abundance of OTUs), then we conducted ANOSIM tests (vegan package in R; R Core Team 2018) to statistically test for differences within each group. (3) We analysed community diversity by calculating Alpha diversity indices (chao, Shannon and Simpson) using Qiime (ver. 1.9.1) and the t-test or Wilcoxon test was used to compare the differences within each comparison group.

3. Results

A total of 5254 operational taxonomic units (OTUs) were identified from cloacal samples of 24 Kentish plovers (17 adults and 7 juveniles) and 9 lesser sand plovers (7 adults and 2 juveniles) encompassing 36 bacterial phyla, with prevalence and abundance of specific phyla differing among individuals (Fig. 1a). The dominant phylum in both populations show consistency, with the most abundant phyla being Firmicutes (60% in Kentish plovers, 59% in less sand plovers), Proteobacteria (14% in Kentish plovers, 15% in less sand plovers) and Bacteroidetes (10% in Kentish plovers, 14% in less sand plovers), making up the core microbiota (Fig. 1b).

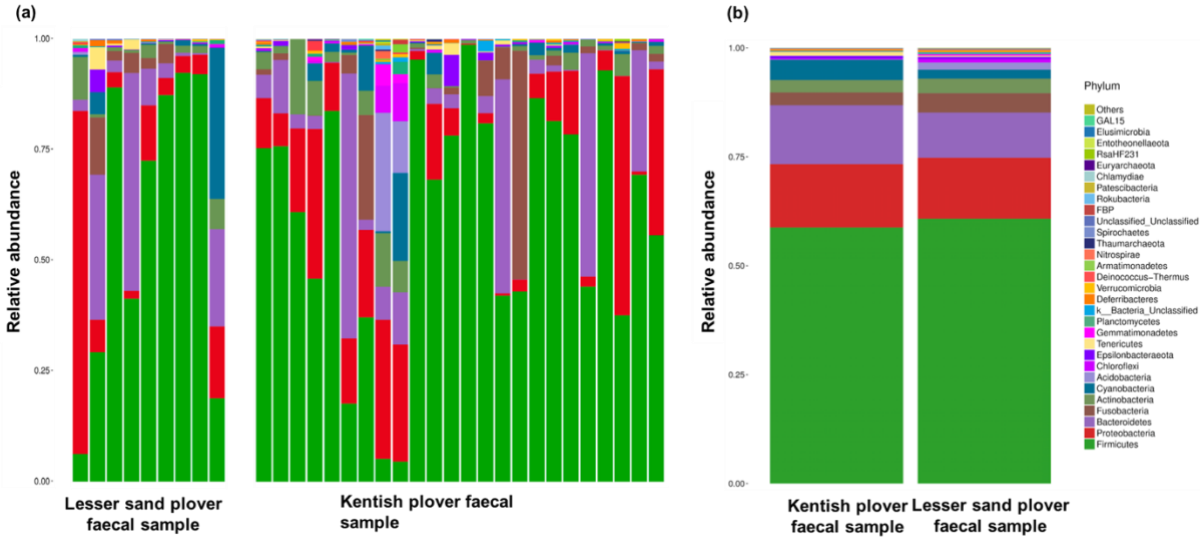

Figure 1 a. Stacked bar chart of the relative abundance of top 30 bacterial phyla in the faecal microbiota of 24 Kentish plovers and 9 lesser sand plovers. **b.** Stacked bar chart of the average relative abundance of bacterial

phyla in faecal microbiota of Kentish plovers and lesser sand plovers. Phyla in the legend are listed in order of increasing abundance.

3.1 Differences in bacteria taxa

Kentish plover vs. lesser sand plover

The faecal microbiota of Kentish plover consisted of 3848 OTUs compared to 1406 OTUs for less sand plovers, of which 1208 were shared between two populations (Fig. 2a). Of the total number of OTUs found, 2640 were unique to Kentish plovers, and a much smaller number of OTUs (n = 198) were unique to lesser sand plovers.

5 OTUs (2 Bacteroidetes; 2 Acidobacteria; 1 Firmicutes; 1 Proteobacteria) were significantly enriched and 1 OTU (1 Proteobacteria) was significantly reduced in Kentish plover faecal samples (Fig. 3a). We also found that the relative abundance of bacteria genus of Corynebacterium_1, Euzebya and Ruminococcaceae_UCG-008 showed significant difference between Kentish plovers and lesser sand plovers (Fig. 4a-c, $P_{\text{Corynebacterium\_1}} = 0.04$, $P_{\text{Euzebya}} = 0.02$, $P_{\text{Ruminococcaceae\_UGC-008}} < 0.01$; Metastats).

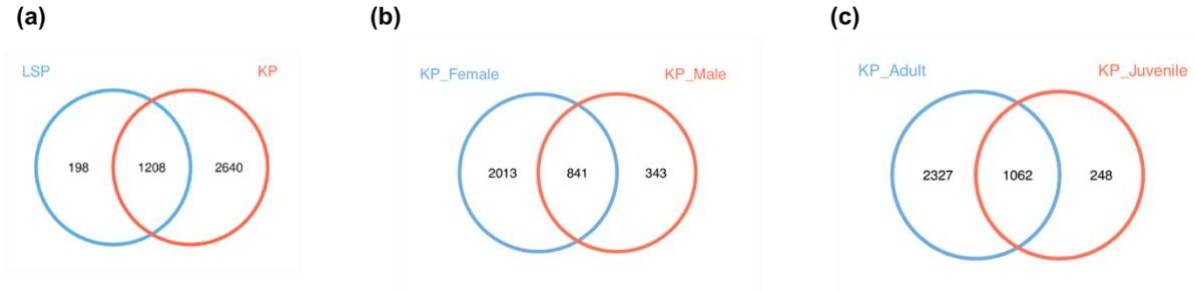

Figure 2. Venn diagrams demonstrating number of shared and unique OTUs in **(a)** Kentish plovers (KP, red) and lesser sand plovers (LSP, blue), **(b)** in female (blue) and male (red) Kentish plover, and **(c)** in adult (blue) and juvenile (red) Kentish plovers.

Female Kentish plover vs. male Kentish plover

Female Kentish plover faecal microbiota consisted of 2854 OTUs, and the number in male Kentish plovers is 1184 OTUs. 841 OTUs were shared between sexes (Fig. 2b). A total 2013 OTUs were found unique to females, and 343 to males.

9 OTUs (4 Proteobacteria; 3 Firmicutes; 1 Gemmatimonadetes; 1 Actinobacteria) were found more abundant in female Kentish plovers and 1 OUT (Firmicutes) in males (Fig. 3b). At a finer scale, we found that the relative abundance of bacteria genus of Acinetobacter showed significant difference between female and male Kentish plovers (Figure 4d, $P_{\text{Acinetobacter}} = 0.01$).

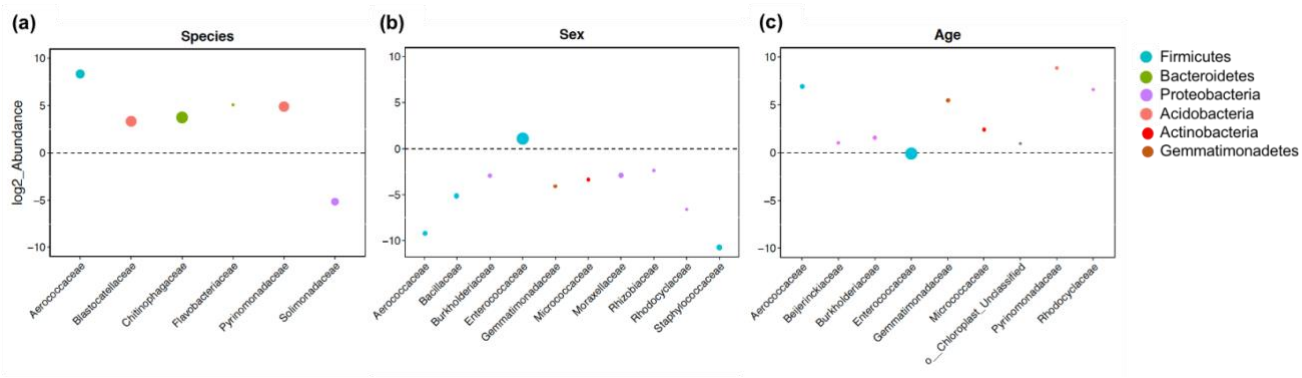

Figure 3. Fold changes for OTUs (circles) that significantly differed between **(a)** Kentish plovers and lesser sand plovers, **(b)** female and male Kentish plovers and **(c)** adult and juvenile Kentish plovers. OTUs below the dashed line are more abundant in lesser sand plovers in (a), female Kentish plovers in (b) and juvenile Kentish plovers in (c). OTUs are grouped by family, coloured by phyla, and sized by mean relative abundance across samples.

Adult Kentish plover vs. juvenile Kentish plover

The number of OTUs in adult Kentish plover faecal microbiota is 3389, and the number in juvenile Kentish plovers is 1310. 1062 OTUs were shared between adults and juveniles (Fig. 2c). 2327 OTUs were unique to adults compare to 248 for juveniles.

7 OTUs (3 Proteobacteria; 1 Firmicutes; 1 Gemmatimonadetes; 1 Actinobacteria; 1 Acidobateria) were found more abundant in adult Kentish plovers and 1 OUT (Firmicutes) in juveniles (Fig. 3c). From genus level, the relative abundance of the genera of Butyricicoccus, Exiguobacterium, Lactococcus and Serratia showed significant difference between adult and juvenile Kentish plovers (Fig. 4e-g, $P_{\text{Butyricicoccus}} = 0.02$, $P_{\text{Exiguobacterium}} = 0.02$, $P_{\text{Serratia}} = 0.03$).

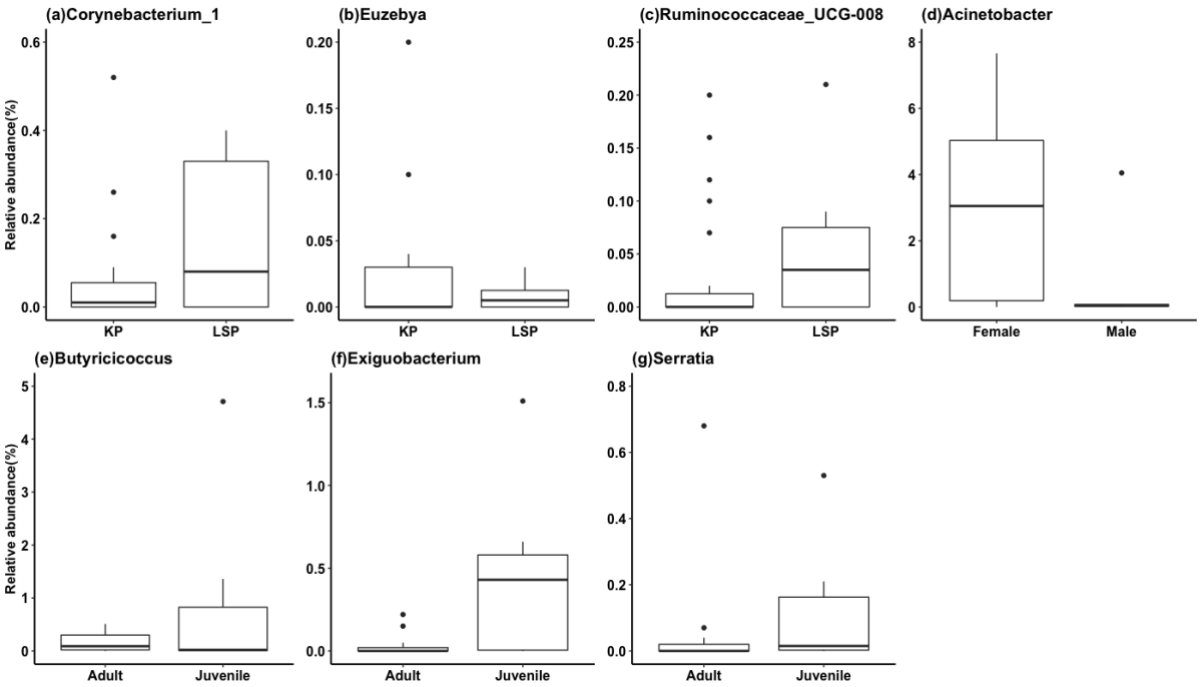

Figure 4. Relative abundance of bacterial genus in the faecal microbiota between Kentish plovers and lesser sand plovers (**a-c**), female and male Kentish plovers (**d**), and adult and juvenile Kentish plovers (**e-g**).

3.2 Differences in community-wide abundance and species diversity

Our results showed that Beta diversity showed no significant difference between each comparison group when using Bray-Curtis (Species: $R = 0.14$, $P = 0.10$; Sex: $R = -0.01$, $P = 0.47$; Age: $R = -0.05$, $P = 0.62$; ANOSIM).

Alpha diversity did not differ between each comparison group, either. For between species: Chao index ($P = 0.37$, Wilcoxon test), Shannon index ($t = -0.04$, $df = 31$, $P = 0.97$, t-test) and Simpson index ($t = -0.44$, $df = 31$ $P = 0.66$, t-test). For between sexes: Chao index ($P = 0.67$, Wilcoxon test), Shannon index ($t = 1.79$, $df = 22$, $P = 0.09$, t-test) and Simpson index ($t = 0.94$, $df = 22$, $P = 0.36$, t-test). For between age groups, Chao index ($P = 0.66$, Wilcoxon test), Shannon index ($t = 0.66$, $df = 22$, $P = 0.51$, t-test) and Simpson index ($t = 0.12$, $df = 22$, $P = 0.90$, t-test).

4. Discussion

In this staged study, due to the limited knowledge in *Charadrius* plover gut microbiota, we first investigated the gut microbiota in two sympatric *Charadrius* plover populations that breed in high altitude environment. We found that at the phylum level, the most abundant phyla in Kentish plover and lesser sand plover faeces are Firmicutes, Proteobacteria and Bacteroidetes, accounting for nearly 90% of the total gut microbiota. This result was consistent with previous studies of the gut microbiota in other avian species (Roggenbuck et al. 2014, Hird et al. 2015, Grond et al. 2018). Firmicutes are associated with mass gain and immune function in mammals and birds, playing an important role in increasing nutrient uptake and metabolic efficiency (Flint et al. 2012, Zhang et al. 2015, John and Mullin 2016), whereas Bacteroidetes is proposed to play a specific role in break-down of cellulose and other plant materials (Thomas et al. 2011, Kohl et al. 2014). However, the function of Proteobacteria in birds remains undetermined. It is also noteworthy that a considerable abundance of Fusobacteria was seen in Kentish plover (relative abundance 4.4%) and lesser sand plover (relative abundance 2.9%) faecal samples. Fusobacteria are often studied in the context of pathogenicity, and in carnivorous birds, Fusobacteria seem to be beneficial for the resistance against pathogens (Roggenbuck et al. 2014, Mendoza et al. 2018). Therefore, it is an interesting avenue for future study to investigate the occurrence of Fusobacteria in shorebirds.

Although the abundance of the dominant phyla did not differ significantly in any of the comparison groups, some bacterial families and genera differ in abundance across comparison groups (Fig. 3 and Fig. 4). At family level, we noticed that the Aerococcaceae family shows consistent biased across the comparison groups, however, we found that this is because one Kentish plover (adult, female) sample contained extremely high level of Aerococcaceae (8.5%), whereas the numbers in the other samples were lower than 0.03%, which skewed the results. Similar patterns were found among Burkholderiaceae distribution in age and sex groups. Such sharp variation in abundance could be the result of sample contamination but it is also possible that the sample size was too small to explain the evident abundance variations in some bacterial taxa. At the genus

level, we found that Corynebacterium and Ruminococcaceae were more abundant in lesser sand plover than in Kentish plover (Fig. 4a-b). Corynebacterium genus is believed to be more abundant in migratory than conspecific residents in *Calidris* shorebirds, it may enable migrating shorebirds to maximise fat deposition and/or energy harvest during migration (Risely et al. 2018), whereas the abundance of Ruminococcaceae has been linked with the maintenance of gut health (Zheng et al. 2019). Therefore, it is possible that life history traits such as migration and the health status of individuals may also account for the different abundance in some bacterial genus.

Our results also showed that within each comparison group, Alpha diversity and Beta diversity showed no difference, suggesting individuals from each comparison group had similar and overlapping gut microbial communities. First, gut microbial composition did not significantly differ between Kentish plovers and lesser sand plovers, suggesting that gut microbiota composition is probably influenced more by environmental factors than genetic factors. We posit that similarity in gut microbiota composition between Kentish plovers and lesser sand plover may stem from the similar range of diets and habitats experienced by both species prior to sampling (David et al. 2014, Hird et al. 2014, Carmody et al. 2015). However, our proposition needs to be tested by further research so as to better understand the role of environment and genetic factor in shaping gut microbiota. For example, to compare the gut microbiota within same species but from different ambient environments as well as account for the influence of wintering ground in shaping gut microbiota (Risely et al. 2018). Furthermore, Ley et al. (2018) suggest that gut microbial communities more similar among closely related species, as Kentish plover and lesser sand plover populations in this study are not only sympatric, but also genetically related. Therefore, another possible aspect for future research is to involve more species that are less genetically related to each other but share common environment. In this study, it would be beneficial to compare gut microbiota of Kentish plovers and lesser sand plovers with other sympatric avian species (e.g. geese, passerines).

Second, within Kentish plovers, sex seems to show no influence on gut microbiota. Studies of human and other mammalian species show that males and females differ in reproductive physiology and behaviour, which may manifest as different gut microbial profiles (Neuman et al. 2015, Aivelo et al. 2016, Grond et al. 2018). However, there were no sex-related differences in gut microbiota in Kentish plovers. The Kentish plover population in this study is monogamous, indicating a similar social behavioural pattern between males and females, the homogeneity in gut microbiota suggests that fundamental biological difference between sexes may show a weaker influence on gut microbiota than ecological environment. In addition, a study shows that mating behaviours could cause transient change in gut microbiota in black-legged kittiwakes *Rissa tridactyla* (White et al. 2010), indicating that the time of sampling might also influence the gut microbiota (e.g. pre-mating vs post-mating), therefore, repeated sampling is required in future work to better understand the sex-difference in gut microbiota.

Third, we did not see any age-related differences in gut microbiota in Kentish plovers either, although studies investigating microbiota in precocial or altricial birds showed that the gut microbiota composition of chicks is markedly different from that of adult birds (González-Braojos et al. 2012, Grond et al. 2017).

However, it is worth noting that chick samples are richer in some bacterial genus than adults, for example, Exiguobacterium and Serratia (Fig. 4f-g). Most Exiguobacterium and Serratia are non-pathogenic environmental bacteria, with the former has ability to survive in varying temperature extremes (Vishnivetskaya et al. 2009, Khanna et al. 2013). This suggests that although the community-wide abundance and species diversity show no difference between adults and juveniles in this study, the development stage of juveniles should be an important factor to be accounted for, since the microbial colonization in shorebird chicks starts soon after hatching, and changes throughout development (Grond et al. 2017). In addition, as is mentioned above, limited sample size and sample contaminations may also conceal the real pattern of gut microbiota between sexes and age groups.

In summary, this study described and compared the gut microbiota of Kentish plovers and lesser sand plovers. The preliminary results showed that the similar breeding environment may have caused homogeneous gut microbiota patterns between the species. Within Kentish plover, gut microbial diversity showed no difference between sex or age group. This study provides baseline information and methodologies that can be used in future studies to better understand the broad-scale patterns in gut microbiota and function in wild birds and assessed how gut microbiota of shorebirds relates to genetic, environmental factors, population social structure, immune function, and disease-related processes.

5. Future directions

Given this study is a phased study, for future study can be carried out from following aspects:

(1) To expand the research scale by including more species and populations under natural conditions across a wide geographic range. This will improve our very limited understanding of gut microbiota in wild birds (especially shorebirds).

(2) The comparative approach will allow us to better assess the aspects of gene, environment and social structure that influence gut microbiota of birds under natural conditions. Therefore, except for broadening sampling, social behavioural monitoring is required.

(3) Much of the functional roles of gut microbial communities in wild birds remain unclear. The application of metagenomics, metatranscriptomics and metaproteomics on elucidating the functional potential of gut microbial communities in previous studies would offer valuable insights for avian gut microbe dynamics in future research (Pérez-Cobas et al., 2013; Kato et al., 2014). More importantly, the pathogenic functional profiles of gut microbial community are essential for our understanding of the link between social structure and disease prevalence, and has significant implications in conservation biology.

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Appendix 6 | Parasites of the Shiny Cowbird, *Molothrus bonariensis*, and the Austral Blackbird, *Curaeus curaeus*, (Passeriformes: Icteridae) in Chile

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Parasitas do chupim *Molothrus bonariensis* e do pássaro-preto-austral *Curaeus curaeus* (Passeriformes: Icteridae) no Chile

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Abstract

Comparative studies of parasites in sympatric bird species have been generally scarce. Parasitic infection/transmission can be spread in a number of ways that suggests possible direct and indirect, horizontal transmission between avian hosts. In order to determine whether two sympatric icterids from Central and Southern Chile share their parasite fauna (ecto- and endoparasites), we examined parasites of 27 Shiny Cowbirds, *Molothrus bonariensis*, and 28 Austral Blackbirds, *Curaeus curaeus*, including individuals captured in the wild and carcasses. We found that Shiny Cowbirds were infected with the chewing lice *Brueelia bonariensis*, *Philopterus* sp. 1, the feather mites *Amerodectes molothrus*, *Proctophyllodes* spp. (species 1 and 2), and the helminths *Mediorhynchus papillosus*, *Plagiorhynchus* sp., *Dispharynx nasuta* and *Tetrameres paucispina*, while Austral Blackbirds had the chewing lice *Myrsidea* sp., *Philopterus* sp. 2, the feather mites *Proctophyllodes* sp. 3, *Amerodectes* sp., and three helminths: *Anonchotaenia* sp., *Capillaria* sp. and *M. papillosus*. The flea *Dasypsyllus* (*Neornipsyllus*) *cteniopus* was found only on the Austral Blackbird. The only parasite species shared by both icterids was the acanthocephalan *M. papillosus*, possibly due to their feeding on the same intermediate insect hosts. With the exception of *B. bonariensis* and *Philopterus* sp. 1 found on the Shiny Cowbird, all species reported in this study represent new parasite-host associations and new records of parasite diversity in Chile.

Keywords: Parasite diversity, sympatry, Icteridae, Phthiraptera, Acari, roundworm.

Resumo

Estudos comparativos de parasitas em espécies de aves simpátricas são escassos. A infecção/transmissão de parasitas pode acontecer de diversas maneiras, incluindo possível transmissão direta, indireta ou horizontal entre as aves hospedeiras. Com o objetivo de determinar se dois icterídeos simpátricos do centro e sul do Chile compartilham a sua fauna parasitária (ecto- e endoparasitas), foram examinados os parasitas de 27 chupins *Molothrus bonariensis* e 28 pássaros-pretos-austral *Curaeus curaeus*, incluindo indivíduos capturados com rede de neblina e em carcaças. Nos chupins analisados, foram encontrados os piolhos de penas *Brueelia bonariensis*, *Philopterus* sp. 1, os ácaros *Amerodectes molothrus*, *Proctophyllodes* spp. (espécie 1 e 2), e os helmintos *Mediorhynchus papillosus*, *Plagiorhynchus* sp., *Dispharynx nasuta* e *Tetrameres paucispina*. Em contraste, os pássaros-pretos-austral estavam infectados com os piolhos *Myrsidea* sp., *Philopterus* sp. 2, os ácaros *Proctophyllodes* sp. 3,

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Appendix 7 | Gastrointestinal and ectoparasites of plumbeous rail, *Pardirallus sanguinolentus* (Aves: Rallidae) in Central Chile

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Original Article

Gastrointestinal and ectoparasites of plumbeous rail, *Pardirallus sanguinolentus* (Aves: Rallidae) in Central Chile

Gastrointestinais e ectoparasitas do saracura-do-banhado, *Pardirallus sanguinolentus* (Aves: Rallidae) do Chile central

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Abstract

With the aim to identify the parasite fauna of plumbeous rail, *Pardirallus sanguinolentus* (Aves: Rallidae) in Chile, 26 carcasses were parasitologically necropsied. The present study revealed the presence of 14 species of parasites (inverse Simpson index = 4.64; evenness = 0.332), including ectoparasites: feather mites: *Analloptes megnini*, *Grallobia* sp., *Grallolichus* sp., *Megniniella* sp., and *Metanalges* sp.; the feather lice *Pseudomenopon meinertzhageni*, *Rallicola andinus*; and *Fulicof ula* sp.; and six species of gastrointestinal helminths: *Heterakispsophiae*, *Porrocaecum ardeae*, *Tetrameres* sp., *Capillaria* sp., *Diorchis* sp., and *Plagiorhynchus* sp. The relatively high parasite richness that was found could be attributed to the highly favorable conditions of wetlands for parasite development. All parasites found, except feather lice, are new records for plumbeous rail. A checklist of parasites for plumbeous rail is presented.

Keywords: Parasites, helminths, diversity, wetlands, rallids.

Resumo

Com o objetivo de identificar a fauna parasitária do saracura-do-banhado, *Pardirallus sanguinolentus* (Aves: Rallidae) no Chile, 26 carcaças foram necropsiadas. O presente estudo revelou a presença de 14 espécies de parasitos (índice Simpson inverso = 4,64; equitatividade = 0,332), incluindo os ácaros de penas: *Analloptes megnini*, *Grallobia* sp., *Grallolichus* sp., *Megniniella* sp. e *Metanalges* sp.; os piolhos de penas *Pseudomenopon meinertzhageni*, *Rallicola andinus* e *Fulicof ula* sp.; e seis espécies de helmintos gastrointestinais: *Heterakispsophiae*, *Porrocaecum ardeae*, *Tetrameres* sp., *Capillaria* sp., *Diorchis* sp. e *Plagiorhynchus* sp. A riqueza parasitária relativa encontrada pode ser devido às condições altamente favoráveis das zonas úmidas para o desenvolvimento do parasita. Todos os parasitos encontrados, com exceção dos piolhos de pena, são novos registros para o saracura-do-banhado. Um checklist dos parasitos do saracura-do-banhado é apresentado.

Palavras-chave: Parasitos, helmintos, diversidade, zona úmida, ralídeos.

Introduction

Parasite-host associations reveal valuable information about the host that should always be considered for studies of biodiversity and conservation (PÉREZ-PONCE DE LEÓN & GARCÍA-PRIETO, 2001), particularly since parasites have been linked to important variations in biodiversity, population declines, and even species extinction (e.g., JOHNSON et al., 1999; CUNNINGHAM & DASZAK, 1998). The plumbeous rail, *Pardirallus sanguinolentus* (SWAINSON, 1838), is one of three members of the genus *Pardirallus* Bonaparte, 1856, in South America. Six subspecies are recognized, three of which are partially distributed in Chile. This bird inhabits all types of wetlands, including brackish and

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Appendix 8 | Gastrointestinal and external parasitism in the Magellanic Horned Owl *Bubo magellanicus* (Strigiformes: Strigidae) in Chile

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Original Article

Gastrointestinal and external parasitism in the Magellanic Horned Owl *Bubo magellanicus* (Strigiformes: Strigidae) in Chile

Parasitos gastrointestinais e externos da coruja-orelhuda *Bubo magellanicus* (Strigiformes: Strigidae) do Chile

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Abstract

To describe the parasitic community of the Magellanic Horned Owl, *Bubo magellanicus* (Aves, Strigiformes), 19 carcasses from central Chile were analyzed. Ectoparasites were collected through plumage inspection, while endoparasites were collected through traditional techniques of parasitological necropsy. Sixteen owls were infected with at least one species of ectoparasite (84.21%) or endoparasite (31.58%). Eleven of 19 birds (57.89%) harbored feather mites of the three species *Pandalura cirrata* (42.11%), *Glaucalges attenuatus* (47.37%), and *Kramerella* sp. (10.53%), whereas 16 individuals (84.21%) harbored the chewing louse *Strigiphilus chilensis*. Only six birds (31.58%) were infected with helminths; the nematodes *Capillaria tenuissima* (26.32%) and *Dispharynx nasuta* (5.26%); the acanthocephalan *Centrorhynchus spinosus* (5.26%); and the trematode *Neodiplostomum* sp. (5.26%). Apart from *S. chilensis*, all parasites comprised new records for *B. magellanicus*.

Keywords: Birds, parasites, Phthiraptera, Acari, helminths.

Resumo

Para descrever a comunidade parasitária de coruja-orelhuda *Bubo magellanicus* (Aves, Strigiformes), foram analisados 19 carcaças das aves do centro do Chile. Os ectoparasitos foram coletados inspecionando-se a plumagem e os endoparasitas extraídos por meio de técnicas tradicionais de necropsia parasitaria. Dezesseis corujas estavam infectadas com pelo menos uma espécie de ectoparasito (84,21%) ou endoparasito (31,58%). Onze de 19 aves (57,89%) abrigavam nas penas ácaros de três espécies: *Pandalura cirrata* (42,11%), *Glaucalges attenuatus* (47,37%) e *Kramerella* sp. (10,53%), enquanto que 16 indivíduos (84,21%) estavam parasitados pelo piolho *Strigiphilus chilensis*. Apenas seis aves (31,58%) estavam infectadas com helmintos; os nematoides *Capillaria tenuissima* (26,32%) e *Dispharynx nasuta* (5,26%); o acantocéfalo *Centrorhynchus spinosus* (5,26%); e o trematódeo *Neodiplostomum* sp. (5,26%). Excetuando-se *S. chilensis*, todos os parasitos incluíam novos registros para *B. magellanicus*.

Palavras-chave: Pássaros, parasita, Phthiraptera, Acari, helmintos.

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